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THE EXCITATION OF SPINAL NEURONES BY THE IONOPHORETIC APPLICATION OF AGENTS WHICH CHELATE CALCIUM

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THE importance of a low but constant concentration of calcium ions in the extracellular fluid bathing nerve cells and fibres is now well established and the experimental evidence points to a connection between the degree of membrane calcification and its permeability to sodium and potassium ions (BRINK, 1954; HODGKIN, 1958; SHANES, 1958; MULLINS, 1959). The location of mammalian spinal neurones makes it difficult to alter the ionic composition of the extracellular fluid around them. However, by the recent techniques of electrophoretic injection into the environment of cells, compounds can now be applied which specifically raise or lower the concentrations of certain extracellular ions.

The anions of certain acidic amino acids excite the spinal neurones of the cat (CURTIS, PHILLIS and WATKINS, 1960). Although these acids themselves complex calcium ions only very weakly, it is noteworthy that the structurally related compounds of the Versene group are the most powerful of the known calcium complexing agents. It was of interest therefore to study the effects of these and other sequestering agents upon spinal neurones. The substances were chosen so as to cover a wide range both in their structure and in the stability of the complexes which they form with calcium ions. The reason for this selection was to render easier a differentiation of effects due primarily to the chelation of free extracellular calcium ion from those, if any, due to a direct interaction of the substances with membrane components. This latter mechanism probably accounts for the excitant and depressant actions of amino acids related to glutamic and γ-amino-*n*-butyric acids respectively (cf. CURTIS and WATKINS, 1960).

METHODS

As described previously (CURTIS, PHILLIS and WATKINS, 1959), all experiments were carried out upon neurones within the lower lumbar and upper sacral segments of spinal cats lightly anaesthetized with pento-barbital-sodium. The substances were applied into the vicinity of interneurones, motoneurones and Renshaw cells by the ionophoretic technique, co-axial, double-barrel, or five-barrel electrodes being used. These cells were identified by their position in the spinal cord and by their responses to dorsal and ventral root volleys. Motoneurones have characteristic spike potentials in response to dorsal or ventral root volleys and are unable to follow high frequency repetitive stimulation. Interneurones respond repetitively to single afferent volleys but fail to react to stimulation of the ventral root. In contrast Renshaw cells are fired repetitively at high frequency by ventral root volleys and occasionally respond to impulses reaching the spinal cord via the dorsal roots. Responses were usually recorded extracellularly but in the case of motoneurones intracellular records were obtained by means of the central barrel of the co-axial pair (cf. CURTIS *et al.*, 1959). For a comparison

of the effects with those of the excitatory amino acids, the complexing agents were usually prepared for use in the electrodes as saturated solutions in aqueous NaOH solution, the final pH being adjusted to approximately 8. Solutions of higher pH values (10–11) were occasionally used with the weaker chelating agents since the greater charge on the substance at the higher pH increases the conductance of the solution and thus reduces the resistance of the electrode containing it. This in turn permitted the use of relatively large ionophoretic currents without introducing the complication of electrode noise due to the passage of current through high resistance electrodes coupled capacitively with the recording electrode. As with amino acid solutions at these high pH values (CURTIS and WATKINS, 1960), co-axial electrodes prepared immediately beforehand were used to reduce the possibility of damage to the glass by the alkaline solution. Citrate and oxalate ions were passed from saturated solutions of the potassium salts, these being more soluble than the corresponding sodium salts. In no instance was any effect observed when either sodium or potassium ions were passed into the environment of cells. The substances used are listed in Table 1 together with their structure and relevant data concerning their calcium chelating properties. All have been compared by their action upon interneurones (column 8) and 0 indicates the absence of effect. The symbol + indicates an excitatory action, the number of symbols expressing the relative intensity of such effect. This rating is approximate and depends upon a comparison of effects produced by equal ionophoretic currents upon the same or similar spike potentials (see later).

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RESULTS

(A) *EDTA*

It is convenient initially to present results obtained with ethylenediamine-NNN′N′-tetra-acetic acid (EDTA), for the action of this substance was typical of all those chelators with observable effects. As with certain acidic amino acids (CURTIS *et al.*, 1960) this substance evoked spike potentials of both interneurones and Renshaw cells and, when applied from the outer barrel of a co-axial electrode to the exterior of a motoneurone impaled with the inner electrode, was shown to produce a similar membrane depolarization. For example the responses of Fig. 1A were recorded from a motoneurone by means of an electrode containing 0·6 M-K_2SO_4 solution which was the 'inner' barrel of a co-axial pair (Fig. 1E). A direct coupled amplifier was used and the resting potential of the cell was −57 mv. The full line of Fig. 1C indicates the ionophoretic current flowing through a saturated solution of the sodium salt of EDTA of pH 8, contained in the outer barrel of this co-axial electrode. Initially a cationic current of 50 mμA was flowing which prevented the diffusion of the active anion from the electrode. When this current was increased to 520 mμA (first arrow of Fig. 1C) the 'membrane' potential was decreased by 5 mv, a value which was maintained whilst this current was flowing irrespective of its duration. A similar potential difference was recorded when both electrodes were in an extracellular position (Fig. 1B) and when currents of the same size were passed in either direction (Fig. 1D), and represents the potential generated by current flowing in the material separating the two tips, in this particular case the resistance being 10 kΩ. This potential was therefore not actively generated by the neuronal membrane and if EDTA had no effect on the neurone, a mirror image of this potential would be expected (broken line of Fig. 1A) when the ionophoretic current was reversed. However, when this was done (second arrow of Fig. 1C) an active membrane depolarization of 10 mv was observed after a latency of 20 sec. The action of EDTA was determined upon six different motoneurones and in each case a depolarization was observed which had a latency of 5–20 sec, depending partly upon the current used. Thus with currents approaching 500 mμA the latency was shorter than in the case of the depolarization occurring with 100 mμA. However, in no case was the latency as short as that observed

with glutamate and aspartate which also cause a membrane depolarization when applied under similar circumstances (CURTIS *et al.*, 1960).

It is not unreasonable to consider that the activation of the spike-generating mechanism of neurones is a direct consequence of a membrane depolarization which reaches threshold (cf. ECCLES, 1957), and that repetitive excitation is the result of a depolarization which is maintained above the threshold level. Consequently it can be

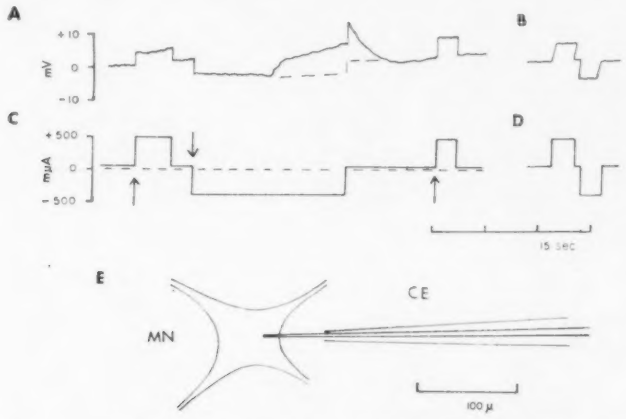

FIG. 1. A–D.—Records obtained by means of a co-axial electrode (CE) from a motoneurone (MN), the probable situation being illustrated in E.

A—alterations in membrane potential recorded by the inner barrel which contained 0·6 M-K_2SO_4. This is a tracing from an ink recorder following amplification with a direct-coupled amplifier. The resting potential (Zero level) was −57 mV. Depolarization of the membrane shown as a positive potential.

C—current passed through the outer barrel, which contained a solution of EDTA at pH 8, recorded simultaneously with A. Zero current indicated by the broken line, there being a cationic (positive) backing current of 50 mμA. At the first arrow this was increased to 520 mμA and at the second, reversed to an anionic current of 420 mμA. The third arrow signals the re-application of the cationic current. When such currents were maintained for up to 60 sec no further alteration in membrane potential occurred.

B and D—As for A and C respectively but the inner electrode was in an extracellular position. The potentials (B) were generated by the current flow in the resistive material between the tips of the electrodes.

Calibrations mV for A, B;
mμA for C, D;
15 sec for A–D
100 μ for E.

assumed that, as with motoneurones, the excitatory action of EDTA upon interneurones and Renshaw cells is also the result of a depolarization, this leading on to the activation of the spike-generating mechanism in the case of the smaller cells. With motoneurones, the depolarization produced by EDTA was usually insufficient to initiate a spike, as was also the case with the depolarization of these cells produced by the excitant amino acids (CURTIS *et al.*, 1960). The spike potentials of Fig. 2 were recorded from a single cell of the dorsal horn over a period of 26 seconds. This cell was fired repetitively by a volley in the tibial nerve and this orthodromic stimulus (marked with an arrow in Fig. 2A) was applied throughout the series at approximately one second intervals. With the sweep speed used, the individual spike responses cannot be clearly distinguished. A cationic current initially prevented anions from diffusing out of the electrode and at ▲ in Fig. 2A an anionic current of 100 mμA

passed EDTA into the environment of the cell. The cell began to fire at low frequency 5–6 sec later and this firing progressively increased in frequency throughout the 19 sec during which the chelator was applied. Characteristically the firing occurred in bursts, which later tended to fuse (Fig. 2D). When the current was terminated, firing ceased rapidly within 400 msec. The presence of orthodromic test volleys did not affect the responses of these cells to the chelating agent. The orthodromic responses of the cell in Fig. 2 were increased by only one or two spikes by EDTA but other cells were observed in which the facilitation was greater.

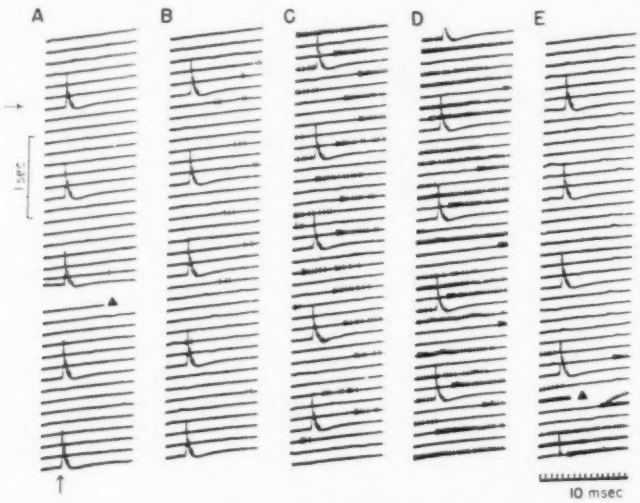

FIG. 2.—Spike potentials recorded extracellularly by one barrel of a double-barrelled electrode near a single dorsal horn interneurone and photographed upon film moving parallel to the Y axis of the oscilloscope, the speed being indicated by the time marker to the left of column A. There was less than 5 msec between sweeps and approximately every second the cell was activated orthodromically by a volley in the tibial nerve, the first volley being indicated by an arrow in Fig. 2A. The series commences at the bottom of column A and the columns follow sequentially, the top of one being continuous with the bottom of the next. At ▲ in A an anionic current of 100 mμA passed EDTA from the other barrel of the electrode, this current terminating at ▲ in E. Time—10 msec.

The action of EDTA upon both interneurones and Renshaw cells differs slightly from that of glutamic acid. Characteristically, the excitant action of glutamic acid begins after a brief latency of about 200–500 msec and ceases on the termination of the applying current within a similar interval. When large currents are used the cells are initially discharged at a high rate and are subsequently blocked, presumably owing to the neuronal membrane being rendered incapable of further support of spike potentials (cf. CURTIS *et al.*, 1960).

When single neurones, either interneurones or Renshaw cells, were observed with multibarrel electrodes a comparison could be made between the actions of the excitant amino acids and EDTA. Fig. 3 illustrates typical results, the noteworthy difference between these records being the relatively long latency preceding the action of EDTA. After an initial period within the latter portion of which there was often slight accentuation of the orthodromically evoked spikes, the neurones responded in short high-frequency bursts. Thereafter the action of EDTA resembled that of glutamate in

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being dependent upon the ionophoretic current. If low currents were used, these bursts were maintained, ceasing rapidly within 200–500 msec of the termination of the current. If, however, larger currents were used (Fig 3), the bursts merged and the cell was fired at high frequency. The spike potential then suffered a series of changes in shape and eventually became blocked. A slight difference between glutamate and

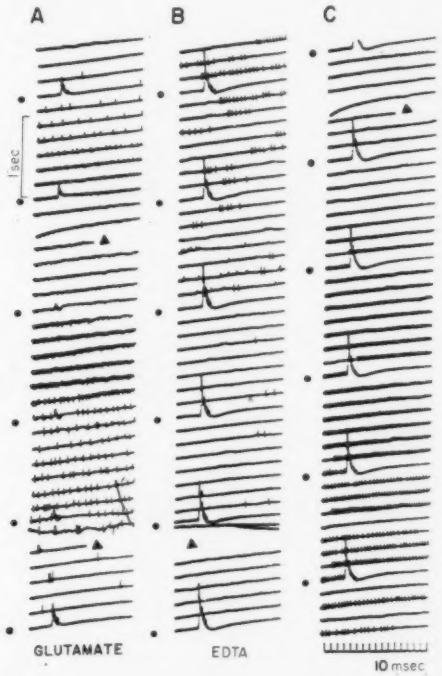

FIG. 3.—Spike potentials from an interneurone, recorded in a fashion similar to those of Fig. 2 but by means of the central barrel of a five-barrelled electrode. As in Fig. 2 the columns begin at the bottom.

A—response to glutamic acid passed as an anion from one barrel of the electrode, the ionophoretic current of 110 mμA flowing between the two symbols ▲.

B and C—continuous record showing the effect of EDTA which was applied with an anionic current of 200 mμA flowing through another barrel of the electrode and which began at ▲ in B and ceased at ▲ in C. Throughout both series the cell was stimulated orthodromically every second by a volley in the peroneal nerve, the sweeps during which this occurred have a filled circle to the left.

Calibration. Vertical—1 sec indicating the speed of the film upon which the responses were recorded; horizontal—10 msec.

EDTA was occasionally observed in the manner in which the spike potentials changed shape under the influence of these two substances. Typically, glutamic acid converted negative-positive spikes into predominantly positive spikes before the spike potential was completely abolished (Fig. 3A). The chelators, on the other hand, usually appeared to diminish both the negative and positive phases of the spike potential and as the negative portion was usually the greatest in magnitude, small negative spikes were recorded before blocking occurred. In some cases, however, the progressive deterioration of the spike followed closely that observed for glutamic acid, blocking being preceded by small positive spikes. With both the excitant amino acids

and the chelators, the synaptically evoked spikes were also blocked, and recovered shortly after the termination of the ionophoretic current.

The long latency of EDTA action was characteristic of all the active chelators. It could be reduced by applying the substances with higher currents but was never as brief as that with the amino acids. This effect is probably not entirely due to the slower rise in the concentration of the active substance at the neuronal surface because

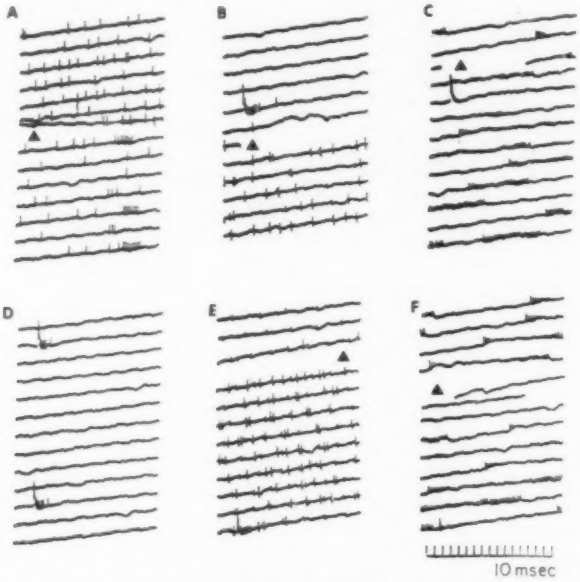

FIG. 4.—Extracellular spikes of a Renshaw cell, recorded by the central barrel of a five-barrel electrode in response to the ionophoretic application of acetylcholine (A, D); glutamic acid (B, E) and EDTA (C, F) passed at different times from individual barrels of the electrode before (A–C) and whilst (D–F) dihydro-β-erythroidine was applied to the cell. As in Figs. 2 and 3, the spikes were recorded on moving film, the lower traces of each column preceding those above. The symbols ▲ in A, B, C, E and F indicate the termination of the respective ionophoretic currents. This cell was also fired synaptically by a ventral root volley in B, C and D. Time—10 msec.

when very low currents were used to apply the anions of glutamic or aspartic acids, these agents completely failed to excite cells unless effects were observed within 1–3 sec. The latency of firing by chelators could be shortened by certain procedures. For example, simultaneous application of two chelators, one of which may even have been inactive, or only weakly active, by itself, diminished the time in which the other excited the cell. Thus, application of citric acid as an anion diminished the latency of firing produced by EDTA. Similarly, repeated applications of EDTA at frequent intervals resulted, in a reduction in the latency of firing.

As with the excitant amino acids (CURTIS *et al.*, 1960), the extracellularly recorded focal potentials produced by the activity of many interneurones could be reduced in size and even converted to positive potentials by the application of EDTA. Pharmacological tests were applied in order to investigate the site of action of the chelators when they were applied to single cells. Certain neutral amino acids depress spinal interneurones (CURTIS *et al.*, 1959), and the application of one of these, γ-amino-*n*-butyric acid, into the vicinity of single cells readily prevented their excitation by

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EDTA. Further, an analysis of the excitation of Renshaw cells by both acetylcholine and glutamate showed that dihydro-β-ertyhroidine (DHE), which readily blocks the cholinergic excitation of these cells, does not affect the spikes evoked by the amino acid ion (CURTIS *et al.*, 1960). Similar findings were observed for EDTA, as illustrated in Fig. 4. This cell was fired repetitively by a ventral root volley (Fig. 4B) and responded to acetylcholine (Fig. 4A), glutamate ion (Fig. 4B), and EDTA (Fig. 4C) when these were passed ionophoretically from different barrels of a five-barrel electrode. Dihydro-β-erythroidine was then passed from another barrel as a cation, a current of 200 mμA being used. During this application, the same ventral root stimulus was less effective and acetylcholine failed to excite the cell (Fig. 4D). However, glutamate and EDTA, with the same ionophoretic currents as in the control series of Fig. 4B and C, produced exactly the same excitatory action. It can, therefore, be concluded that these substances act upon the Renshaw cells independently of the receptors specialized for acetylcholine. The synaptic or ionophoretic cholinergic excitation of Renshaw cells is blocked by procaine (CURTIS and ECCLES, 1958). This substance also blocks the orthodromic spikes of dorsal horn cells and the excitation of these cells and of Renshaw cells by the excitant amino acids (CURTIS and PHILLIS, 1960). Similar results were obtained when these cells were fired by EDTA, this excitant action also being suppressed by procaine.

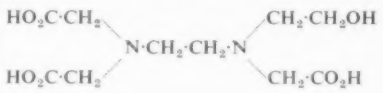
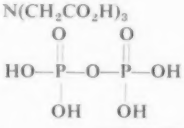
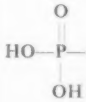
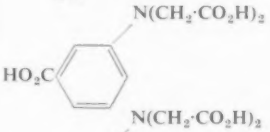
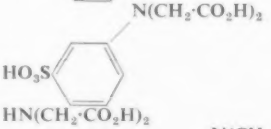
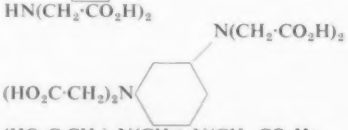
(B) *Other substances*

The first eighteen compounds have been arranged in Table 1 in the order of their ability to chelate calcium ions at physiological pH (see Discussion). When any of the first twelve were passed ionophoretically into the region of single interneurones, excitation not unlike that already described occurred, whereas substances 13 to 24, apart from oxalic acid (19) and aspartic acid (23), were inert. The method of ionophoretic application from double-barrel electrodes does not allow an accurate assessment of the relative potency of a series of compounds. However, a comparison can be based upon the latency of onset and frequency of firing when the same current is used to pass the different substances. In addition, four substances could be compared when they were passed from separate barrels of a five-barrel electrode upon one cell. In this instance however, care had to be taken that the current injecting any one substance was not also passing another from an adjacent barrel.

With these methods and ionophoretic currents of 100 mμA, there was little quantitative difference among the first eight substances (Table 1). Nitrilotriacetic acid and pyrophosphoric acid had slightly weaker excitant actions, whereas citric acid and 2:2-bis[di(carboxymethyl)amino]diethyl sulphide were only weak excitants. When currents of 200 mμA were applied, the latter two chelators were more effective; furthermore the excitant action of oxalic acid (19) became apparent. This substance was inert when currents of 100 mμA were used to apply it to cells having extracellularly recorded spike potentials of approximately 1 mV. Currents greater than 200 mμA were impracticable because of the generation of electrical noise in the electrode and also because such currents often influenced the cellular responses independently of the ion being applied. The substances have been graded (Table 1, column 8) according to their excitant action with currents of 100–200 mμA, oxalic acid being a very weak excitant.

EDTA was the only chelator which was passed ionophoretically upon the surface

TABLE 1.—EFFECT OF CALCIUM CHELATING

	1	2
No.	Substance	Structure
1	Ethylenediamine-NNN′N′-tetra-acetic acid (EDTA)	$(HO_2C{\cdot}CH_2)_2N{\cdot}CH_2{\cdot}CH_2{\cdot}N(CH_2{\cdot}CO_2H)_2$
2	DL-Propylene-1: 2-diamine-NNN′N′-tetra-acetic acid[6]	$(HO_2C{\cdot}CH_2)_2N{\cdot}CH(CH_3){\cdot}CH_2{\cdot}N(CH_2CO_2H)_2$
3	D-Propylene-1: 2-diamine-NNN′N′-tetra-acetic acid[6]	
4	L-Propylene-1: 2-diamine-NNN′N′-tetra-acetic acid[6]	
5	1: 2-Bis[2-di(carboxymethyl)amino-ethoxy]ethane	$(HO_2C{\cdot}CH_2)_2{\cdot}N(CH_2)_2O(CH_2)_2O(CH_2)_2N(CH_2{\cdot}CO_2H)_2$
6	2: 2′-Bis[di(carboxymethyl)amino] diethyl ether	$(HO_2C{\cdot}CH_2)_2{\cdot}N(CH_2)_2{\cdot}O(CH_2)_2{\cdot}N(CH_2{\cdot}CO_2H)_2$
7	N′-(2-Hydroxyethyl)ethylenediamine-NNN′-triacetic acid[7]	
8	Mg-EDTA Complex (Mg salt)	
9	Nitrilotriacetic acid	$N(CH_2CO_2H)_3$
10	Pyrophosphoric acid[9]	
11	Citric acid[9]	$HO_2C{\cdot}CH_2{\cdot}C(OH)(CO_2H){\cdot}CH_2CO_2H$
12	2: 2′-Bis[di(carboxymethyl)amino] diethyl sulphide	$(HO_2C{\cdot}CH_2)_2N{\cdot}(CH_2)_2{\cdot}S{\cdot}(CH_2)_2N(CH_2{\cdot}CO_2H)_2$
13	Phosphoric acid[9]	
14	*m*-Carboxyphenyliminodiacetic acid	
15	*m*-Sulphophenyliminodiacetic acid	
16	Iminodiacetic acid	$HN(CH_2{\cdot}CO_2H)_2$
17	1: 3-Diamino*cyclo*hexane-NNN′N′-tetra-acetic acid	
18	1: 8-Octamethylenediamine-NNN′N′-tetra-acetic acid	$(HO_2C{\cdot}CH_2)_2N(CH_2)_8N(CH_2{\cdot}CO_2H)_2$
19	Oxalic acid	$HO_2{\cdot}C{\cdot}CO_2H$
20	Malonic acid	$HO_2C{\cdot}CH_2{\cdot}CO_2H$
21	Succinic acid	$HO_2C{\cdot}CH_2{\cdot}CH_2CO_2H$
22	Glutaric acid	$HO_2C{\cdot}CH_2{\cdot}CH_2{\cdot}CH_2{\cdot}CO_2H$
23	Aspartic acid	$HO_2C{\cdot}CH_2{\cdot}CH(NH_2){\cdot}CO_2H$
24	Ca-EDTA complex (Na salt)	

(1) Acid dissociation constants and stability constants from BJERRUM, SCHWARZENBACH and SILLEN (1957, 1959). In most cases they are those which were determined in physiological saline or in media of similar ionic strength. Values selected are for temperatures nearest to 37°.

(2) L = ligand and is the entity in terms of which the equilibrium constants are expressed.

(3) The 'excess constant', E, is defined by equations (1) and (2)—see text.

(4) C expresses the molar concentration of chelator necessary to complex 90% of a 1·25 mM-calcium solution i.e. $P = 9$ (See Text).

(5) The symbol + indicates excitation; 0 indicates no excitation of interneurones.

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AGENTS ON SPINAL NEURONES

3	4				5[1]		6	7	8	9
Type[2] (H_nL)	Acid dissociation constant[1] pK_a				Calcium complex		E[3]	Conc[4]	Action[5]	No.
	1	2	3	4	Type	Log K_1				
H_4L	1·99	2·67	6·16	10·26	CaL^{2-}	10·57	$2·07 \times 10^{-8}$	$1·12 \times 10^{-3}$	+++	1
H_4L					CaL^{2-}		$2·07 \times 10^{-8}$	$1·12 \times 10^{-3}$	+++	2
H_4L					CaL^{2-}		$2·07 \times 10^{-8}$	$1·12 \times 10^{-3}$	+++	3
H_4L					CaL^{2-}		$2·07 \times 10^{-8}$	$1·12 \times 10^{-3}$	+++	4
H_4L	~2	2·68	8·85	9·46	CaL^{2-}	11·00	$3·35 \times 10^{-8}$	$1·12 \times 10^{-3}$	+++	5
H_4L	1·75	2·76	8·84	9·47	CaL^{2-}	10·05	$2·99 \times 10^{-7}$	$1·13 \times 10^{-3}$	+++	6
H_3L	2·64	5·33	9·73		CaL^{-}	8·0	$2·17 \times 10^{-6}$	$1·14 \times 10^{-3}$	+++	7
($Mg L^{2-}$)	—	—	—	—	CaL^{2-}	10·57	See Text	$1·25 \times 10^{-3}$	+++	8
H_3L	1·89	2·49	9·73		CaL^{-}	6·41	$8·36 \times 10^{-5}$	$1·88 \times 10^{-3}$	++	9
H_4L		2·52	6·08	8·45	CaH_2L	5	$2·38 \times 10^{-4}$	$3·28 \times 10^{-3}$	++	10
								———	—	
H_4L	2·94	4·14	5·82		$CaHL^{-}$	3·2	$6·48 \times 10^{-4}$	$6·96 \times 10^{-3}$	+	11
H_4L	1·75	2·52	8·38	9·42	CaL^{2-}	6·21	$6·80 \times 10^{-4}$	$7·24 \times 10^{-3}$	+	12
								———	—	
—	—	~7	~12		CaHL	1·86	$\sim 1·93 \times 10^{-2}$	$\sim 1·75 \times 10^{-1}$	0	13
H_3L	2·40	4·06	5·10		CaL^{-}	1·46	$3·47 \times 10^{-2}$	$3·13 \times 10^{-1}$	0	14
H_3L		2·36	4·79		CaL^{-}	1·26	$5·50 \times 10^{-2}$	$4·96 \times 10^{-1}$	0	15
H_2L		2·65	9·38		CaL	2·59	$2·48 \times 10^{-1}$	2·23	0	16
H_4L	1·77	2·57	8·55	10·91	CaL^{2-}	4·77	$8·32 \times 10^{-1}$	7·5	0	17
H_4L	1·95	2·75	9·94	10·75	CaL^{2-}	4·51	$2·4 \times 10^{-1}$	216	0	18
H_2L	1·2	4·2			CaL	~3[8]	$\sim 10^{-3}$	$\sim 1 \times 10^{-2}$	(+)	19
H_2L	2·69	5·24			CaL	1·4	$3·98 \times 10^{-2}$	$3·6 \times 10^{-1}$	0	20
H_2L	4·07	5·28			CaL	1·2	$6·31 \times 10^{-2}$	$5·7 \times 10^{-1}$	0	21
H_2L	4·21	5·06			CaL	1·06	$8·71 \times 10^{-2}$	$7·8 \times 10^{-1}$	0	22
H_2L	1·94	3·70	9·62		CaL	1·6	4·19	37·8	+++	23
							See Text	See Text	0	24

(6) pK_a and log K_1 values not known but taken to be identical to those of EDTA for the calculation of the 'excess constant'.

(7) Isolated from a commercial solution of the trisodium salt ('Versenol 120', Dow Chemical Co., Michigan, U.S.A.)

(8) Extrapolated to zero ionic strength.

(9) For meaning of log K_1 in these cases see BJERRUM *et al.* (1957, 1959). In these special cases, where the ligand is $H_xL^{(n-x)-}$, the excess constant does not have the value given by equation (1) which is valid only where the ligand is L^{n-}. The excess constant for these substances is most simply obtained by individual calculation.

of motoneurones whilst observations were made upon the intracellularly recorded responses of the cells (cf. Fig. 1). However, the similarity of the excitatory actions of all of these agents on interneurones suggests that the mechanism of action is the same in each instance. Thus it can be postulated that all produce a membrane depolarization which, if of sufficient magnitude, evokes a spike response of the cell. In common with EDTA, the excitant chelating agents evoked spikes after a latency of 5–20 sec and this can be correlated with the latency preceding the depolarization of motoneurones by EDTA.

The Ca and Mg complexes of EDTA (Table 1: 24 and 8 respectively) were among those applied ionophoretically near interneurones. The first was passed from a solution of the Na salt, the second from that of the Mg salt. The Ca complex was inert whereas the Mg complex was an excitant with an action only slightly less than that of EDTA itself. Application of Ca^{++} and Mg^{++} from saturated aqueous solutions of the nitrate and chloride respectively was also attempted. Despite the low electrode resistance (20–40 mΩ) currents larger than 40–50 mμA could not be passed through these solutions without the generation of considerable noise. Consequently the extracellular level of these two ions was unlikely to have been greatly increased. Mg^{++} had no effect upon the orthodromic responses of interneurones whereas Ca^{++} appeared to have a very slight depressant action, reversibly reducing the number of synaptically evoked responses.

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DISCUSSION

A variety of isolated tissues has been subjected to experimental investigations in which the extracellular Ca^{++} concentration has been varied. In some there has been no significant alteration in the resting membrane potential (WEIDMANN, 1955; SHANES *et al.*, 1959) whilst in others there was a direct relationship between this potential and the external Ca^{++} concentration (ISHIKO and SATO, 1957; JENERICK and GERARD, 1953; DALTON, 1959; JENERICK, 1959; STAMPFLI and NISHI, 1956; GOSSWEILER *et al.*, 1954). In all instances however, a decrease in Ca^{++} concentration led to a lowered threshold of excitability and even spontaneous activity whereas an increase in the concentration to a certain level resulted in a raised threshold (HERTZ 1947; FRANKENHAUESER, 1957; FRANKENHAUESER and HODGKIN, 1957; FRANKENHAUESER and MEVES, 1958).

The excitant action upon spinal neurones of a series of compounds with known ability to chelate Ca^{++} immediately suggests that such action is associated with a localized lowering of the extracellular concentration of this ion. The excitation is due to a membrane depolarization and, therefore, the action of these compounds is very similar to that which occurs when glutamate, aspartate and cysteate ions are applied ionophoretically to these cells (CURTIS *et al.*, 1960). It has been established that a certain structural arrangement of the amino and acidic groups is necessary for such acidic amino acids to be excitants (CURTIS and WATKINS, 1960). The presence of similar groups in the molecules of some of the active chelators suggests the possibility that these combine directly with 'receptors' located on the membrane as has been postulated for the excitant amino acids. However, such groups are absent in two of the excitant chelators, pyrophosphoric acid and citric acid (Table 1: 10 and 11), but are present in several inactive chelators (Table 1: 14, 15, 16, 17, and 18). This fact, together with the observation that whilst the Ca complex of EDTA (Table 1: 24) was

inactive the Mg complex (Table 1: 8) was a strong excitant, excludes such a participation of the amino acid receptors in the case of the chelators. In addition, all of the excitant chelators were active after a delay of 5–20 sec whereas the amino acids excited cells with a latency which was usually less than 1 sec.

If the action of these substances depended upon their lowering of the extracellular Ca^{++} concentration to a definite proportion of the normal value, it would be expected that their excitatory action would be directly related to the comparative concentrations of each which would be required for this reduction. The method by which they were applied precludes an accurate knowledge of the concentration obtained, but it can be assumed that with the same ionophoretic currents similar distributions of concentration were obtained around the electrode tip (cf. Appendix). In addition, when the responses of different cells were compared, spikes of approximately the same size were observed. Under these conditions, it can be assumed that the electrode was at approximately the same distance from each of the cells being investigated and that as a consequence, the substances were all being tested under similar conditions of concentration. This was particularly so when five-barrel electrodes were used, allowing the application of different agents with identical currents at the same distance from a single cell. The first eighteen compounds (Table 1) are mostly strong metal chelators and are arranged in descending order of their Ca-chelating potency at physiological pH. Compounds 19 to 22 are dicarboxylic acids of lower Ca-chelating power and are included with aspartic acid (23) solely for comparative purposes. The Table is completed by the Ca complex of EDTA, this substance being obviously incapable of binding any additional Ca^{++}.

The extent to which a certain substance H_nL will chelate a metal ion depends upon the selected pH, upon those ionization constants of the substances which influence the production of the ligand (that ionic species with which the metal becomes chelated) and also upon the stability constant of the metal complex. It may readily be deduced that for any ligand L^{n-}, the concentration (C) of the chelating agent which is required to chelate a definite proportion of a metal ion M^{z+} is given by

$$C = [ML^{(n-z)-}] + \frac{P}{K_1}\left(1 + \frac{[H^+]}{Ka_n} + \frac{[H^+]^2}{Ka_n\, Ka_{(n-1)}} + \cdots + \frac{[H^+]^n}{Ka_n Ka_{(n-1)} \cdots K_1}\right) \qquad (1)$$

where $[ML^{(n-z)-}]$ is the concentration of the complex formed,

P is the ratio of the concentration of the complexed metal ion to that uncomplexed,

K_1 is the stability constant of the particular complex $ML^{(n-z)-}$, and Ka_n, $Ka_{(n-1)}$ etc. are the n^{th}, $(n-1)^{th}$, etc. acid dissociation constants of the chelating agent H_nL. With buffered solutions the expression (1) may be simplified to

$$C = [ML^{(n-z)-}] + PE \qquad (2)$$

where E is a constant which can appropriately be called the 'excess constant' since the term PE represents the concentration of excess (i.e. uncomplexed) chelating agent which is required to maintain the desired complex concentration. As the chelating ability of a series of chelators diminishes, so E increases, hence the reciprocal of E can be taken as an index of chelating ability. The values of E for the substances in Table 1 are listed in column 6; for these calculations a value of 7·4 was taken for the pH of the medium. It is clear that the critical value of E is about 10^{-3}. Substances having a value less than this were able to excite neurones, whereas those in which E exceeded this critical value were inert. Thus, there is a definite relationship between the excitant action of these compounds and their ability to chelate Ca^{++}.

In the presence of an excess of the strong chelating agents, however, not only Ca^{++} but also Mg^{++} would be removed from the extracellular fluid. That this effect is not important is demonstrated by the activity of the Mg-EDTA complex. Ionophoretic application of this substance actually results in

an increase in the local Mg^{++} concentration because of the exchange of this metal with the Ca^{++} in the medium. It, therefore, seems probable that the action of the chelating agents is solely due to reduction of the extracellular Ca^{++} concentration.

The extracellular Ca^{++} concentration of the mammalian spinal cord is unknown but the total Ca^{++} concentration of grey matter is 2·5 mM (MANERY, 1952). This is comparable with the concentration for plasma (2·5 mM) but is double that of the cerebrospinal fluid, which is approximately 1·25 mM (DAVSON, 1956). No figures are available for the proportion of Ca which must be removed before neurones become excited. However, on analogy with the alterations observed in either the threshold or resting potential of other tissues (GOSSWEILER *et al.*, 1954; WEIDMANN, 1955; FRANKENHAUESER, 1957; FRANKENHAUESER and HODGKIN, 1957) it can reasonably be assumed that a four to ten-fold diminution would be sufficient to produce the necessary depolarization of spinal neurones.

By assuming extracellular Ca concentrations of 2·5 mM and 1·25 mM it is possible to calculate a series of concentrations of different substances which would be necessary to produce both ten- and four-fold reductions in these calcium concentrations. These are listed in Table 2 for substances 1, 8, 9, 10, 11, 12, 13, 19, 20, and 24 of Table 1, whilst the values calculated for a ten-fold reduction in a calcium concentration of 1·25 mM are listed for all substances in column 7 of Table 1. With the Mg-EDTA complex, equation (1) is not applicable because of an additional factor arising from the dissociation of this complex. However, the total concentrations which would be required for a given value of P and an assumed Ca^{++} concentration can still be readily calculated to be very close to those estimated for EDTA itself. Clearly, no such concentrations are calculable for the Ca complex of EDTA, this substance being incapable of significantly altering the extracellular Ca^{++} concentration.

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Excitatory action was observed for substances 1–12 and 19, the others being inert apart from aspartic acid (23). The results indicated that nitrilotriacetic acid (9) and pyrophosphoric acid (10) were not as powerful excitants as substances 1–8 whilst citric acid (11) and 2:2′-bis[di(carboxymethyl)amino]diethyl sulphide (12) were weaker in action. Oxalic acid (19) was a very weak excitant and phosphoric acid (13) failed to affect neurones. It may thus be assumed that the concentrations of phosphoric acid obtained were not in the range calculated in Table 2, columns 5–8, whereas those of oxalic acid were just within this range. These observations demonstrate that by means of ionophoretic currents of 100–200 mμA it is possible to achieve in the immediate neighbourhood of spinal neurones a concentration of chelator which probably lies within the range 3–11 $\times$ 10^{-3}M (cf. substances 11, 12 and 19; Table 2), the precise value depending upon the actual extracellular Ca^{++} concentration and the true magnitude of P, both of which are unknown. It is evidently not possible to attain concentrations exceeding this limiting figure. The attainable concentration at any one point, dependent upon both the ionophoretic current and the distance from the electrode tip, becomes maximal when larger ionophoretic currents are impracticable and when closer approach to the cell results in impalement of the cell or in the cell response becoming affected by the current alone. As discussed in the Appendix, steady-state concentrations can be calculated at different distances from an electrode tip passing an ionic species into a uniform medium. Under these ideal conditions, and with an ionophoretic current of 100 mμA, a maximal chelator concentration lying within the range 3–11 $\times$ 10^{-3} M would theoretically be realised within 4–14 μ of the electrode tip. However, the extracellular medium cannot be regarded as uniform, as necessarily assumed for these calculations. In addition the situation is further complicated by the inward diffusion of Ca^{++} into the area affected by chelator, interaction between them at the boundary eliminating both substances in this region.

This is relevant to consideration of the long latency of the excitatory action of the chelating agents upon cells, which is clearly very different from that of the excitatory amino acids (CURTIS *et al.*, 1960). The latter compounds, which chelate Ca^{++} only

very weakly (for example, see aspartic acid, Table 1: 23) manifest their action within 1–3 sec of the commencement of the ionophoretic application, and this is almost certainly associated with their direct combination with 'receptors' upon the membrane. The powerful chelating agents, however, do not evoke firing of interneurones until after a latency of 5–20 sec. Similar current magnitudes were used for both series of compounds and these dissimilar delays were observed when applying amino acids

TABLE 2.—ANALYSIS OF CALCIUM CHELATION

1	2	3	4	5	6	7	8
Substance	No. in Table 1	Activity	E	$C_{9\cdot2\cdot5}$	$C_{3/2\cdot5}$	$C_{9/1\cdot25}$	$C_{3/1\cdot25}$
EDTA	1	+	$2\cdot07 \times 10^{-8}$	$2\cdot25 \times 10^{-3}$	$1\cdot88 \times 10^{-3}$	$1\cdot13 \times 10^{-3}$	$9\cdot4 \times 10^{-4}$
Mg-EDTA	8	+	See text	$2\cdot5 \times 10^{-3}$	$1\cdot93 \times 10^{-3}$	$1\cdot25 \times 10^{-3}$	$9\cdot75 + 10^{-4}$
Nitrilo-triacetic acid	9	+	$8\cdot36 \times 10^{-5}$	$3\cdot0 \times 10^{-3}$	$2\cdot12 \times 10^{-3}$	$1\cdot87 \times 10^{-3}$	$11\cdot9 \times 10^{-4}$
Pyrophos-phoric acid	10	+	$2\cdot4 \times 10^{-4}$	$4\cdot41 \times 10^{-3}$	$2\cdot59 \times 10^{-3}$	$3\cdot28 \times 10^{-3}$	$16\cdot6 \times 10^{-4}$
Citric acid	11	+	$6\cdot48 \times 10^{-4}$	$8\cdot08 \times 10^{-3}$	$3\cdot81 \times 10^{-3}$	$6\cdot96 \times 10^{-3}$	$28\cdot8 \times 10^{-4}$
2:2-Bis [di(carboxy-methyl) amino] diethyl sulphide	12	+	$6\cdot8 \times 10^{-4}$	$8\cdot37 \times 10^{-3}$	$3\cdot91 \times 10^{-3}$	$7\cdot24 \times 10^{-3}$	$29\cdot8 \times 10^{-4}$
Phosphoric acid	13	0	$1\cdot43 \times 10^{-2}$	$1\cdot77 \times 10^{-1}$	$8\cdot98 \times 10^{-2}$	$1\cdot75 \times 10^{-1}$	$8\cdot88 \times 10^{-2}$
Oxalic acid	19	(+)	$\sim 10^{-3}$	$\sim 11 \times 10^{-3}$	$\sim 4\cdot9 \times 10^{-3}$	$\sim 10 \times 10^{-3}$	$\sim 3\cdot9 \times 10^{-3}$
Malonic acid	20	0	4×10^{-2}	362×10^{-3}	122×10^{-3}	361×10^{-3}	121×10^{-3}
Ca-EDTA	24	0	See text				

Column 3 + indicates excitation of interneurones; 0 no action
Column 5 Concentration of chelator to complex 90% of a 2·5 mM-calcium solution
Column 6 Concentration of chelator to complex 75% of a 2·5 mM-calcium solution
Column 7 Concentration of chelator to complex 90% of a 1·25 mM-calcium solution
Column 8 Concentration of chelator to complex 75% of a 1·25 mM-calcium solution

and chelating agents from adjacent barrels of the one electrode (cf. Fig. 3). Under ideal conditions (cf. Appendix) a sphere of radius approximately 17 μ containing no Ca^{++} would be achieved within 1 sec of the commencement of the ionophoretic application of a strong chelating agent. The region of Ca^{++} concentration of between a quarter and a tenth of its bulk level would clearly lie at a somewhat greater distance from the electrode within the same time interval. Thus, although the actual conditions may differ significantly from the ideal, it remains probable that the latency of action of the strong chelating agents involve certain factors in addition to the rate of depletion of Ca^{++} in the extracellular fluid.

In this connection consideration must be given to the probability that Ca^{++} is adsorbed or complexed to the neuronal membrane (SHANES, 1958; MULLINS, 1959;

SHANES *et al.*, 1959). Thus, for a chelating agent to be effective when applied into the extracellular fluid it must not only remove 'free' Ca^{++} from this space but also a certain amount of 'bound' Ca from the membrane. The overt excitant action of these agents would be dependent upon the depletion of membrane or 'bound Ca which in turn will be governed by several factors. The most important of these would probably be the rate of dissociation of the Ca-membrane complex. However, because the membrane Ca may be located within pores which are too small to accommodate molecules as large as the chelators (cf. SHANES, 1958), the rate of diffusion of the dissociated Ca must also be considered. The possibility also exists that internal Ca may be able to migrate to replace Ca lost from a more external situation in the membrane. The rate of depletion of the cellular or membrane reserve of ionic Ca has thus been suggested to be the reason for the slow onset of depolarization evoked in myelinated frog nerve fibres by total Ca deprivation (STAMPFLI and NISHI, 1956). The same factors, together with the rate at which Ca^{++} is cleared from the extracellular space, probably account for the comparatively long latency of action of the chelating compounds. The observation that excitation ceases within 200–300 msec after the termination of the ionophoretic current indicates the rapidity with which, in the absence of continued application of chelating agent, diffusion restores the original concentration of Ca^{++} around the cell and recalcification of the membrane takes place.

Analyses of the action of Ca^{++} upon the permeability of axonal membrane to Na and K have led to certain postulates concerning its function (FRANKENHAUESER and HODGKIN, 1957; SHANES, 1958; MULLINS, 1959). It can be considered that the 'membrane' Ca controls the permeability to these ions and that partial decalcification, which occurs in association with a propagating spike, facilitates their movement across the membrane in the sequential fashion observed for the spike potential. In particular if some of the Ca is removed, the Na 'carrying' system becomes more 'available' at the resting potential of the membrane, thereby lowering the threshold for excitation. In addition, however, low extracellular Ca levels may result in an increased 'resting' permeability towards Na^{+} thereby producing an actual depolarization. It can therefore be postulated that the excitant action of substances selectively removing Ca^{++} from the environment of neurons is due to an influx of Na^{+} into the cells. This produces a depolarization which initiates spike potentials when the threshold is attained. The depolarization could arise because the threshold for 'triggering' the Na 'carrier' mechanism of affected areas of the membrane was lowered, allowing the usually subthreshold excitatory noise (BROCK, COOMBS and ECCLES, 1952) to increase the membrane permeability towards Na in these particular areas. Alternatively, the removal of Ca from the membrane may directly increase this permeability and produce a depolarization.

The prevention of the excitatory action of EDTA by procaine is presumably indicative of the 'stabilizing' action of this substance upon the Na 'carrier' (SHANES *et al.*, 1959; WEIDMANN, 1955; CURTIS and PHILLIS, 1960). The blocking action of the depressant amino acids however is almost certainly associated with the specific increase in membrane conductance produced by these agents in the absence of a membrane potential alteration (CURTIS *et al.*, 1959). The failure of DHE to alter the the excitation of Renshaw cells by chelating agents specifically excludes a mechanism of their action directly involving cholinoceptive receptors.

The action of chelating agents upon the post-synaptic membrane is distinct from

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any effect upon the pre-synaptic terminals. It might be expected, by analogy with observations at the neuromuscular junction (CASTILLO and STARK, 1952; CASTILLO and ENGBACK, 1954; HUTTER and KOSTIAL, 1954; JENKINSON, 1957), that a low extracellular Ca concentration would reduce the amount of transmitter released. Such a factor might explain the occasional failure of chelating agents to facilitate orthodromic activitation of cells (Fig. 3), but it cannot be very important as this synaptic excitation was facilitated more frequently, owing presumably to the concomitant depolarization of the post-synaptic membrane.

SUMMARY

1. A series of compounds with known ability to chelate calcium ions was applied ionophoretically to neurones of the lumbar segments of the spinal cat.

2. Many were found to excite interneurones, Renshaw cells, and motoneurones, this excitant action being related to the chelating action of the substances.

3. This excitation was associated with a membrane depolarization which was observed when one of the series, EDTA, was applied to motoneurones by using co-axial electrodes.

4. The manner in which calcium ions influence neuronal behaviour is discussed and an analysis is made of the theoretical extracellular concentrations obtained when substances are passed electrophoretically from microelectrodes.

APPENDIX

Theoretical Aspects of Electrophoretic Application of Substances from Microelectrodes

The microelectrodes used in this investigation can be considered to be approximately cylindrical over the terminal 500 μ, with an internal diameter at the tip of approximately 2 μ, the wall thicknesss being less than 0·5 μ. The electrodes were filled with concentrated solutions of electrolytes and by applying a suitable potential either cations or anions were injected into the medium around the tip. Because the resistance of an electrolyte varies directly with the distance, inversely with the cross-sectional area and (approximately) with the concentration, it is easily deduced that even when the electrode solution has ten times the specific conductivity of the medium, almost the entire drop in applied potential occurs within the micro-electrode. Hence, as a first approximation, the added ions or molecules will move through the medium only by diffusion. Also, unless there is a mechanical displacement of the electrolyte into the medium, the quantity of the ion M^{z+} or X^{z-} (if these are the only ions present) which passes out of the electrode per second will be $1{\cdot}036 \times 10^{-5}\ ni/Z$ μmoles, where n is the ionic transference number and i the current in μA.

By assuming that the electrode tip is a point source within a uniform medium, the concentration C of the ion M (or of X if the current be anionic) at a distance r μ from the tip of the electrode is given by the expression

$$C \text{ mole/l.} = \frac{8{\cdot}25 \times 10^{-6}\, n\, i}{Z D r} \operatorname{erfc} \frac{r \times 10^{-4}}{2\sqrt{Dt}} \qquad (3)$$

where D is the diffusion coefficient of M (or X) and the uniform current flows for time t seconds. This equation derives from that given by CARSLAW and JAEGER (1959),

erfc x being 1-erf x, where erf x is the error function integral, tables of which are available (Tables of Probability Function, Works Project Association, New York, 1941). If $r \times 10^{-4}$ is not greater than $2\sqrt{Dt}$, equation (3) can be replaced by the following approximation which is correct to within 5%

$$C = \frac{8{\cdot}25 \times 10^{-6}\, ni}{ZDr}\left(1 - \frac{5{\cdot}85 \times 10^{-5}\, r}{\sqrt{Dt}} + \frac{1{\cdot}61 \times 10^{-9}\, r^2}{Dt}\right) \qquad (4)$$

Fig. 5 shows the concentrations calculated from equation (3) for a monovalent ion of

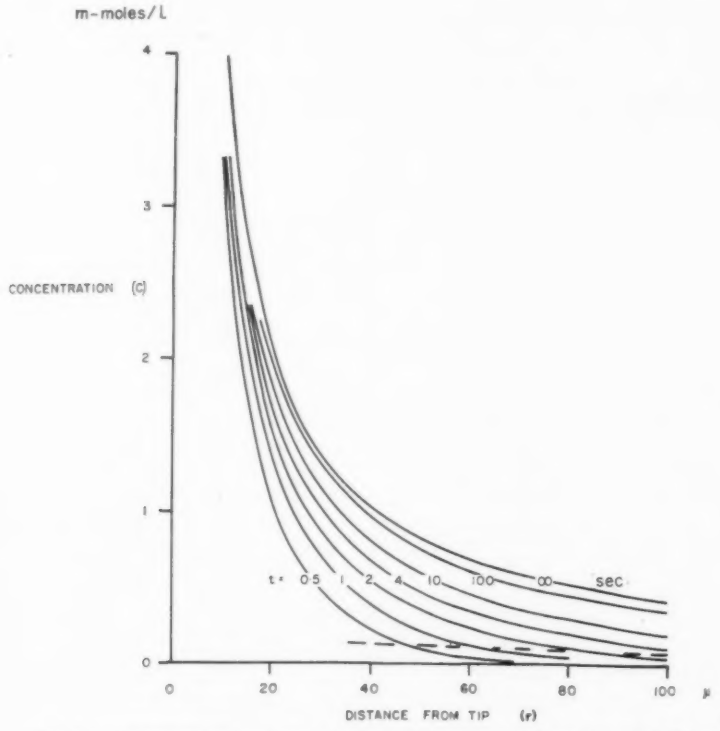

FIG. 5.—Concentration (ordinate), in m-moles/l, of a univalent ion of diffusion coefficient 1×10^{-5} cm²/sec and transference number 0·5 at a distance $r\ \mu$ (abscissa) from the tip of an electrode when passed by an ionophoretic current of 100 mμA. The different curves indicate concentration–distance relationships at the indicated times after the beginning of the ionophoretic current. The broken line indicates those conditions below which equation (4) is insufficiently accurate to be used instead of equation (3), see text.

diffusion coefficient 1×10^{-5} cm^2 sec^{-1} and transference number 0·5, over the range 10–100 μ from the electrode tip after a current of 100 mμA has been flowing for the various times. The upper curve is for the steady-state condition (t very large, erfc approaching unity, so that C varies as $1/Dr$), while the broken line shows the conditions below which equation (4) is insufficiently accurate to be used instead.

To apply equations (3) and (4) for any ionic species it is necessary to know both D and n. Under the experimental conditions (medium equivalent to an 0·16 M-NaCl solution at 37°) it is reasonable to assume that, for any particular added ion, D is

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constant throughout the medium, since the concentration of this ion is small when compared with the total concentrations of ions present (cf. WANG, 1952). If D is not known, a good estimate can be obtained from the Nernst limiting equation, $D_0 = RT\lambda_0/ZF^2$ where RT and F have their usual meaning and λ_0 is the limiting equivalent conductance of the ion. At 37° this reduces to $D_0 = 2{\cdot}76 \times 10^{-7}\ \lambda_0/Z\ \mathrm{cm^2 sec^{-1}}$ and for most of the common ions λ_0 lies between 60 and 100 mhos. Thus, values of D are obtained from $1{\cdot}7 \times 10^{-5}$ to $2{\cdot}8 \times 10^{-5}$ for univalent ions and $0{\cdot}9 \times 10^{-5}$ to $1{\cdot}4 \times 10^{-5}$ for bivalent ions. For large ions, λ_0 tends to a lower limit of about 40. Substitution in equation (3) shows that as a steady-state condition is approached, C varies directly with the ionophoretic current and inversely with λ_0, but is independent of the valency of the ion.

By definition, for an ion in a conducting medium containing a single electrolyte, $n = \lambda/\Lambda$ where Λ is the equivalent conductance of the electrolyte. It is sufficient for the present purposes to use limiting values of λ and Λ, although if desired at least partial correction can be made for the effect of ionic strength (HARNED and OWEN, 1943). By using the Nernst equation and the relation for n, equation (3) becomes

$$C \text{ mole/l.} \approx \frac{30i}{\Lambda_0 r} \operatorname{erfc}\left(0{\cdot}095r\sqrt{\frac{2}{\lambda_0 t}}\right) \tag{5}$$

which is convenient as conductances can be obtained experimentally.

In a number of instances, this ionophoretic technique has also been used to apply species that are zwitterions in neutral solution (CURTIS and WATKINS, 1960; CURTIS *et al.*, 1959, 1960). For example, if the electrode solution is sufficiently acid, glycine is present as the univalent cation and can be passed by ionophoresis into the surrounding medium. However, as the latter is for practical purposes neutral, the cation immediately loses a proton to become the glycine zwitterion. The quantity of the ion passing out of the electrode is still given by $1{\cdot}036 \times 10^{-5}\ ni/Z$ but in the medium it is necessary to know the diffusion coefficient of the neutral species. By extrapolating from results at 1° and 25° for many amino acids, peptides and sugars (LONGWORTH, 1952, 1953), the diffusion coefficient of neutral molecules in water at 37° can be estimated to be $3{\cdot}6 \times 10^{-5}/(M^{1/3} - 1{\cdot}24)$ where M is the molecular weight. The average error is probably less than 10% and for values of M between 60 and 300, D is $1{\cdot}3 \times 10^{-5}$ to $0{\cdot}7 \times 10^{-5}$; i.e. substantially the same as for the larger, singly charged ions.

It is concluded that the passage of any given quantity of current will result, to within a factor of about two, in the same amount of an added species being present at any given distance from an electrode, whether or not the species is a univalent ion, a bivalent ion, or a univalent ion which loses its charge on passing into the medium.

If the viscosity of the medium is significantly greater than that of water, diffusion coefficients will be somewhat less than those calculated above. A possibly more serious source of error lies in neglecting the small electric potential which exists between the tip of the microelectrode and the indifferent electrode which is at the external boundary of the medium. Over the interval from $r\ \mu$ to $(r + \delta r)\ \mu$, measured from the centre of the electrode tip, the increase in electrical resistance is $\rho_m \delta r/4\pi r^2$, where ρ_m is the specific resistivity of the medium. Hence the total resistance (R_m) between the electrode tip and a spherical surface at any distance from it is $\rho_m(2/r_e - 1/r)4\pi$, the factor 2 being inserted because the electrode solution at the tip can be

taken to be hemispherical with a radius of r_e μ. With increasing distance, R_m rapidly approaches a constant value but most of the resistance is found within a short distance of the tip. Thus, for a tip of 1 μ radius, almost 75 per cent of the total resistance would occur within the first μ and 90 per cent within the first 4 μ. From Ohm's law, the voltage drop in the medium is proportional to the resistance and thus, even if the total voltage across it was only 10 mv, the average potential gradient over the first 4 μ, measured from the tip would be 25 v/cm. As most ions have velocities of about 10 μ/sec under a potential gradient of 1 v/cm, this comparatively high potential gradient would make a significant contribution to their outward movement from the region of the tip, causing them to cover the distance of 4 μ within 20 msec. It is essential therefore to keep the ratio of the electrode resistance (R_e) to R_m as large as possible if calculations based on diffusion are to be valid. Since from geometrical considerations, R_e varies as $1/r_e^2$ while R_m varies as $1/r_e$, R_e/R_m is increased by making r_e smaller. Also in practice, diffusion of the more concentrated solution from the electrode will reduce the resistance of the medium in the immediate vicinity of the tip. Under experimental conditions, values of R_m and R_e are about 10 kΩ and 10–100 MΩ respectively, so that for a current of 100 mμA less than 1 mv is applied across the medium. The effect of this voltage is not significant over the range of distances (10–100 μ) which is of interest. The fraction of the total applied potential which is dropped across the medium is, within wide limits, independent of the concentration of the electrode solution.

The part played by electro-osmosis when applying ions from microelectrodes can be assessed by substitution in the equation

$$v = \zeta r_e^2 \varepsilon E / 4\eta l (300)^2 \qquad (6)$$

where v ml is the volume of liquid transported per second by electro-osmosis through a capillary of length 1 cm and radius r_e cm under an applied potential of E volts; ζ in volts is the zeta or electrokinetic-potential, ε is the dielectric constant of water (80) and η is the coefficient of viscosity of the solution. The value of ζ is not known, but it is small at high ionic concentrations and is almost certainly less than 50 mv. By taking this figure as a generous estimate, a value of 2×10^{-10} ml/sec is obtained for v. If the electrode solution is 1 M with respect to the ion to be injected, this would add 2×10^{-7} μmoles of the ion to the medium every second. This quantity is to be compared with 5×10^{-7} μmoles for a univalent ion and $2{\cdot}5 \times 10^{-7}$ μmoles for a bivalent ion which is added as the result of the current flow of 100 mμA. If, as believed, the estimate of the electro-osmotic contribution is too high, these values show that the effect is never as great as, and may be much smaller than, that due to ionophoresis. An effect which has not been considered above is that in aqueous solutions, ions are more or less hydrated so that when there is a differential movement of ions in an electrical field there is also a net movement of water. Because cations are usually more hydrated than are anions, it is possible when cations are being expelled from a microelectrode for them to carry some of the electrode solution with them and in this way 'inert' and uncharged species could be transported into the medium.

The above discussion has been concerned with the passage of ions from an electrode into a uniform medium. In practice these conditions probably do not exist; the extracelular space of the nervous system is considered to be a cleft of approximately 200 Å existing between neurones and neuroglia (HORSTMANN and MEVES,

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1959). The lack of experimental detail makes it of little value to attempt any discussion of diffusional behaviour in such a system, but it seems reasonable to expect that the same general conclusions would be reached, although of course the concentration-distance relationship of Fig. 5 would not hold, the concentration at a particular distance being eventually higher than that calculated. In addition, difficulties arise when there is an interaction between ions in the external medium and those emerging from the electrode, such interaction virtually eliminating both ions from further effectiveness.

In the present case, reaction between a chelating agent and Ca^{++} leads to a local reduction in Ca^{++} concentration and a diffusion of Ca^{++} from the bulk phase towards the depleted area. If the extreme condition is assumed that Ca^{++} and chelating agent react instantaneously to form a non-dissociable 1 : 1 complex, a boundary must be established, inside of which no free Ca^{++} is present and outside of which there is no chelating agent. Also, just outside this boundary Ca^{++} concentration is less than in the bulk phase. There is no simple mathematical equation to describe the motion of this boundary in the case where chelating agent is added at a uniform rate. However, by making the reasonable assumption that inside the boundary the concentration curve for the chelating agent is the same as in free diffusion, it has been deduced (JAEGER J. C., to be published) that the radius, r, of the boundary, t seconds after current begins to flow, is given approximately by

$$r \text{ (in } \mu) = \frac{2 \times 10^4 \sqrt{D_1 t}}{\sqrt{\pi} + 2k\sqrt{D_1 t}} \tag{7}$$

where D_1 is the diffusion coefficient of Ca^{++}, and $k = 4 \times 10^{-3}\pi \cdot C_2 D_1/Q$, where C_2 in moles/l. is the initial Ca^{++} concentration and Q is the amount of chelating agent (moles) passing per second into the solution. Substitution of typical values ($D_1 = 1 \times 10^{-5}$, $C_2 = 0{\cdot}0025$, $Q = 10^{-12}$) shows that, in this case, after 1 sec the boundary is 17 μ from the electrode tip, and, 9 sec later, is 8 μ further away; it never extends more than 32 μ from the tip.

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NUCLEOSIDE METABOLISM IN BRAIN EXTRACTS FROM RAT, RABBIT AND GUINEA PIG*

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ALTHOUGH the action of nucleoside phosphorylase has been clearly elucidated (KALCKAR, 1947, 1950) and its wide distribution in various tissues and organisms has been documented (LEVENE and MEDIGRECEANU, 1911; KLEIN, 1935; STERN *et al.*, 1952; SABLE, 1950; MANSON and LAMPEN, 1951), very little information on the detailed fate of both purine and pentose moieties in brain has been reported. A study, initiated to investigate the products of *in vitro* nucleoside metabolism by brain preparations from rats, rabbits, and guinea pigs, showed that soluble extracts of brain metabolized purine ribosides to form phosphate esters and lactic acid from the pentose moiety, whereas the liberated purines were only deaminated, the ring structure remaining intact.

EXPERIMENTAL

Brain preparations. Whole brain from rat, rabbit, or guinea pig was excised after the animal was bled following ether anaesthesia or stunning by a blow on the back of the neck. The brain was packed in ice, weighed, minced, and suspended in Tris-KCl solution (1 part of M-Tris buffer, pH 7·4, plus 9 parts of isotonic KCl) in proportions of 1 part of tissue by weight to 2 parts of Tris-KCl by volume. In some experiments this proportion was changed to 1 part tissue (wt) and 4 parts Tris-KCl (vol). The tissue was homogenized in the cold by using a Potter-Elvehjem homogenizer with a Teflon pestle (Arthur H. Thomas Co., Cat. No. 4288-B), and the homogenate was centrifuged in the cold at 20,000 **g** for 15 min. The clear supernatant liquid was decanted from the residue and this extract was subsequently used for incubations. In some experiments the whole homogenate was used for incubation.

Incubation and analysis. Incubation mixtures contained 1 ml of brain extract (or homogenate) and 5 μmoles of nucleoside or pentose phosphate, 10 μmoles of [^{32}P] KH_2PO_4 (containing 0·2 mc of [^{32}P] phosphate per 5 ml of 0·050 M-phosphate) at pH 7·4, and 1 μmole $MgCl_2$ in 0·05 M-Tris buffer, pH 7·4, in a total volume of 2 ml. Incubations were carried out under air at 37° in a Dubnoff Metabolic Shaker. The reaction was terminated by cooling the incubation mixture and then adding cold 25% trichloroacetic acid (TCA) to a final concentration of 8%. The supernatant fluid was separated from the insoluble residue, and portions were removed for pentose analysis by the MEJBAUM (1939) orcinol procedure and for paper chromatographic separation of phosphate ester by the RAPPOPORT and CHEN (1960) procedure. Lactic acid was determined by the method of BARKER and SUMMERSON (1941), and proteins were estimated by the colorimetric biuret procedure (GOA, 1953). Absorption spectra of purines and purine nucleosides were measured by using a Beckman recording DK-2 spectrophotometer.

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RESULTS

Extracts from rat, rabbit, and guinea pig brains metabolized guanosine, inosine, adenosine, and ribose-5-phosphate.* Ribose was metabolized to a small extent by guinea pig, and to an even less extent by rabbit, extracts, whereas rat brain extracts showed no activity (Table 1). Deoxyribonucleosides were only slightly metabolized by all three species.

Paper chromatograms of the phosphate esters formed during the incubations with extracts of guinea pig brains were identified as fructose diphosphate, hexose

TABLE 1.—NUCLEOSIDE METABOLISM BY BRAIN EXTRACTS

Substrate	Substrate utilization (%)*		
	Rat	Rabbit	Guinea Pig
Guanosine	79	80	80
Inosine	50	41	58
Adenosine	52	46	58
Ribose-5-phosphate	79	83	60
Ribose	0	10	29
Deoxyguanosine	0	15	20
Deoxyinosine	0	10	12
Deoxyadenosine	0	10	20

* Conditions of incubation as described in the experimental section. Initial concentration of deoxy-ribonucleosides 2·5 μmoles; the rest were 5 μmoles. Incubated for 2 hr.

phosphates (mixture of fructose-6-phosphate and glucose-6-phosphate), ribose-5-phosphate, ribulose-5-phosphate, and 3-phosphoglyceric acid (Fig. 1). Similar data were obtained from experiments with extracts of rat and rabbit brains.

Time studies on the utilization of guanosine and the formation of phosphate esters were made with brain extracts from each of the three species (Fig. 2). Radioautograms similar to the guinea pig results (Fig. 3) were obtained with brain extracts from the other two species. Lactic acid accumulated as one of the end products in concentrations of 0·5 μmoles for each μmole of ribonucleoside incubated for 2 hr. The influence of pH on guanosine metabolism by the brain extracts was constant in the range pH 7·4–9·0 (Fig. 4).

Experiments with brain preparations from rats of different ages (newborn, 10, 20, 30, and 60 days) showed the preparations from newborn animals to have metabolized approximately 10 percent less guanosine, based on fresh weight of tissue, than the preparations from the other age groups. Similar studies were made on selected gross anatomical parts of the rat brain, namely, the cerebellum, cerebral cortex, brain stem, and hypothalamus. In the only two age groups thus studied (10 and 60 days), cerebellum showed a significantly lower utilization of guanosine estimated on the basis of fresh weight of tissue (Table 2).

* P_i, inorganic orthophosphate; R-1-P, ribose-1-phosphate; R-5-P, ribose-5-phosphate; Ru-5-P, ribulose-5-phosphate; Xu-5-P, xylulose-5-phosphate; Ga-3-P, glyceraldehyde-3-phosphate; DHA-P, dehydroxyacetone phosphate; 3PG, 3-phosphoglyceric acid; 2PG, 2-phosphoglyceric acid; DPG, 2:3-diphosphoglyceric acid; HP, hexose phosphates; FDP, fructose-1, 6-diphosphate; F-6-P, fructose-6-phosphate; G-6-P, glucose-6-phosphate; G-1-P, glucose-1-phosphate; DPN, diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; UDPG, uridine diphosphoglucose.

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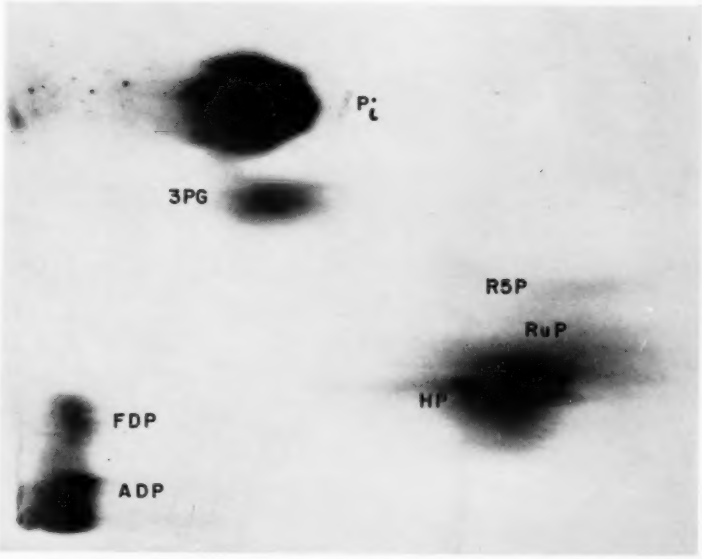

FIG. 1.—A radioautogram of phosphate esters, formed from guanosine incubated with guinea pig brain extracts, separated in two dimensions by paper chromatography.

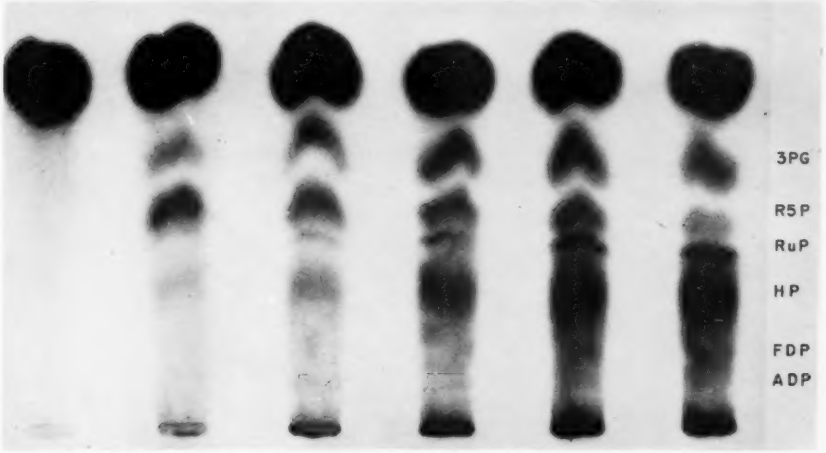

FIG. 3.—A radioautogram of a single dimension paper chromatograph of the phosphate esters formed during a time study of guanosine metabolism by guinea pig brain extracts.

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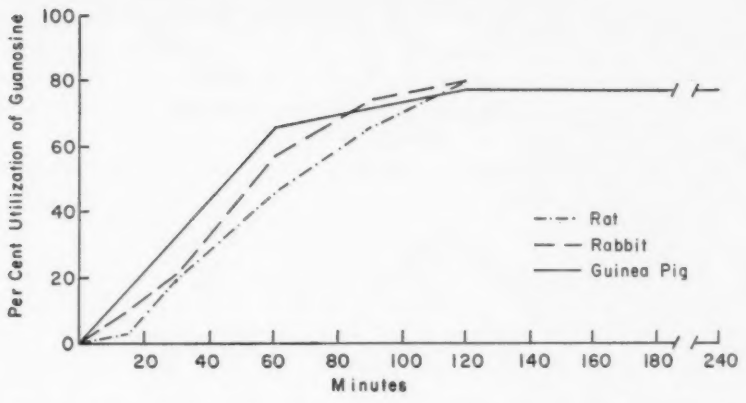

FIG. 2.—Rate of guanosine metabolism by rat, rabbit, and guinea pig brain extracts.

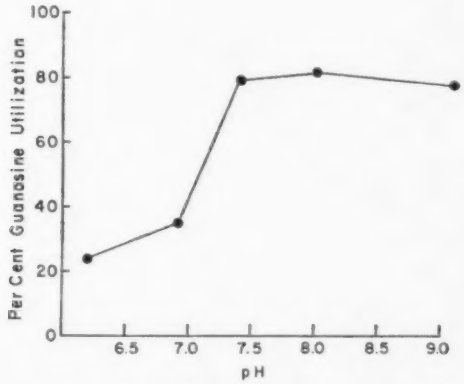

FIG. 4.—Influence of pH on guanosine metabolism by guinea pig brain extracts in 0·05 M-Tris buffer.

TABLE 2.—GUANOSINE METABOLISM BY BRAIN HOMOGENATES* FROM RATS AT DIFFERENT AGES AND FROM DIFFERENT BRAIN PARTS

Age (days)	Percent of added guanosine metabolized				
	Whole brain	Cerebellum	Cerebral cortex	Brain stem	Hypothalamus
Newborn	64	—	—	—	—
10	70	53	76	63	71
20	76	—	—	—	—
30	75	—	—	—	—
60	73	52	76	75	75

* Homogenates consisted of 1 part by weight of fresh tissue and 2 parts 0·1 M-Tris-isotonic KCl, pH 7·4. Incubation conditions as described in Experimental section.

Spectrophotometric examination of the purine products formed from guanosine, inosine, and adenosine incubations indicated that guanosine was converted to xanthine and adenosine to hypoxanthine (Fig. 5). Since the spectral curve for inosine is identical to that of hypoxanthine (the product from inosine incubations), paper chromatograms established the presence of hypoxanthine in these incubations, and this method confirmed the formation of xanthine from guanosine and hypoxanthine

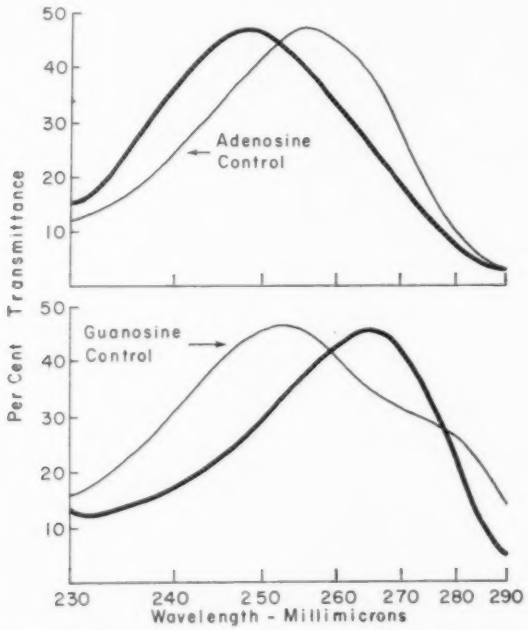

FIG. 5.—The changes in the absorption spectra of guanosine and adenosine during incubations as compared to unincubated controls. Heavy line in the upper graph represents hypoxanthine, and in the lower graph represents xanthine formed during incubation.

from adenosine. These changes in the purine moities were identical in the brain extracts of the three species studied.

DISCUSSION

Metabolism of purine ribonucleosides by brain extracts from rat, rabbit, and guinea pig is similar both in degree of substrate utilization (Table 1) and rate of metabolism (Fig. 2). The same phosphate esters are accumulated during incubation (Fig. 1) with brain extracts from any of the three species.

Brain extracts, like those of other tissues (RAPPOPORT, 1959), metabolized the ribose portion of the nucleosides to form hexose phosphates, ribulose phosphate, phosphoglycerates, and lactic acid (Figs. 1 and 3). The initial reaction which clears the ribonucleoside is catalysed by nucleoside phosphorylase and yields ribose-1-phosphate and free purine (KALCKAR, 1947). The former is converted to ribose-5-phosphate by phosphoribomutase (KLENOW, 1953; GUARINO and SABLE, 1954), which in turn forms ribulose-5-phosphate through the action of phosphoriboisomerase (HORECKER, SMYRNIOTIS and SEEGMILLER, 1951; SABLE, 1952). Thus, the

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formation of ribulose-5-phosphate by brain extracts from ribonucleoside can be accounted for in the early phase of the incubation (Fig. 3). Subsequent conversion of ribulose-5-phosphate to 3-phosphoglycerate and hexose phosphates (Figs. 1 and 3) can be attributed to the successive actions of the transketolase and the glycolytic enzymes in the brain preparations. Fig. 6 illustrates schematically the reactions concerned in the formation of the phosphate esters from ribonucleoside metabolism by brain extracts.

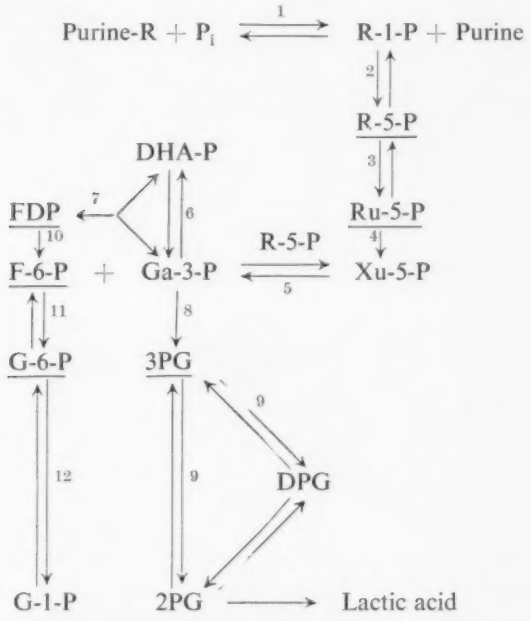

FIG. 6.—Formation of phosphate esters from purine ribosides. Schematic representation of the enzymic reactions involved in the formation of phosphate esters from nucleosides by brain extracts. Underlines indicate esters that have been identified. Enzymes indicated by number are as follows: (1) Nucleoside phosphorylase, (2) phosphoribomutase, (3) phosphoriboisomerase, (4) phosphoketopentose isomerase, (5) transketolase, (6) triosphosphate isomerase, (7) aldolase, (8) triosphosphate dehydrogenase, (9) phosphoglyceric mutase, (10) fructose diphosphatase, (11) phosphoglucoisomerase, (12) phosphoglucomutase.

ROBINS, SMITH and MCCAMAN (1953) studied nucleoside phosphorylase of monkey brain and concluded that phosphoribomutase was absent. The present data on nucleoside metabolism by rat, rabbit, and guinea pig brain extracts indicate that phosphoribomutase is present in these preparations.

A preliminary study of purine riboside metabolism by monkey (*Macaca cynomolgus* (*M. irus*) *Philippinensis*) brain extracts showed that pentose utilization was 74, 44, 42, and 82 percent from guanosine, adenosine, inosine, and ribose-5-phosphate, respectively, in 2 hours' incubation. These data strongly suggest that phosphoribomutase is present in monkey extracts.

From Fig. 5 it appears that the brain extracts should actively metabolize ribose-5-phosphate (SABLE, 1952), an intermediate in ribonucleoside metabolism. Results from incubations of this pentose phosphate with rat, rabbit, and guinea pig brain extracts show that it was readily metabolized (Table 1).

The sequence in which the phosphate esters are formed is illustrated in Fig. 3 where guanosine was incubated with guinea pig extracts. In 15 min the radioautogram showed a very strong spot representing ribulose-5-phosphate, a prominent spot of 3-phosphoglyceric acid, and faint spots of ribose-5-phosphate and hexose phosphate. On prolonged incubation these spots intensified; at 60 min, fructose diphosphate appeared. In 90 min all the esters reached their maximal concentrations. At 120 min the ribulose-5-phosphate spot showed a much lower radioactivity, 3-phosphoglycerate also was reduced in activity, whereas the remaining esters did not exhibit much change in radioactivity as compared to the 90 min incubation products. These changes during incubation of the phosphate esters formed from nucleosides illustrate the dynamic sequence in the reactions following the formation of ribose-1-phosphate from purine ribonucleoside.

The accumulation of xanthine from incubations of guanosine, and hypoxanthine from inosine and adenosine (Fig. 5), indicates the absence of xanthine oxidase in brain extracts (WESTERFIELD and RICHERT, 1949).

The initial tests on the metabolism of guanosine in brain extracts from growing rats showed that only the newborn had a lower activity than the older rat brain preparations. Also, the cerebellum showed a lower guanosine utilization than either cerebral cortex, brain stem, or hypothalamus (Table 2). To establish differences in nucleoside metabolism by brain extracts among different age groups or different gross anatomical sections, it is necessary to determine rates of nucleoside utilization. The initial data presented above represent a measure of enzymic activity after 2 hr incubation, at a time when these metabolic activities have reached a plateau value and only large differences in activity can be observed.

Pentose and hexose phosphates produced in nucleoside metabolism may serve other functions, such as in the formation of coenzymes or intermediates (DPN, TPN, or UDP-glucose, MCILWAIN, 1957) or in the formation of tissue components such as mucopolysaccharides or glycolipids (ROSSITER, 1957). The purines resulting from this nucleoside dismutation are either reconstituted to nucleosides or removed via the circulatory system for complete oxidation to urea by liver or other body tissues.

SUMMARY

Brain extracts from rat, rabbit, and guinea pig metabolize guanosine, adenosine, and inosine in the presence of inorganic phosphate to form phosphate esters and lactic acid via the transketolase and glycolytic enzymes. Xanthine is formed from guanosine and hypoxanthine from adenine or inosine during incubations. The sequence of reactions involved is discussed.

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BRAIN DIAPHORASES*

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WE HAVE previously reported on the localization and some of the properties of enzyme systems which oxidize reduced pyridine nucleotides without added acceptors and which are associated with subcellular particles obtained from rat cerebral cortex (GIUDITTA and STRECKER, 1959.) These enzyme systems were defined in terms of sensitivity to antimycin A and amylobarbitone and are probably analogous to the cytochrome-linked systems which have been studied extensively in heart and liver (*cf.* LEHNINGER, 1951; DE DUVE *et al.*, 1955; ERNSTER and LINDBERG, 1958). Considerable information on the sequence of action and the components of the respiratory chain has been gained from experiments with added oxidation-reduction indicators such as methylene blue. More recently Vitamin K and other quinones have been implicated in the electron transport system of heart and liver mitochondria (MARTIUS and STRUFE, 1954; CRANE *et al.*, 1957). The relationship between the activity of heart and liver mitochondria with added acceptors and the diaphorase originally described in 1937 and subsequently (DEWAN and GREEN, 1937; ADLER, EULER and HELLSTRÖM, 1937) has not been completely clarified.

Our own studies of electron transport in brain preparations have also shown that quinones and oxidation reduction indicators are able to oxidize reduced pyridine nucleotides. However, in contrast to the difficulty observed in solubilizing systems showing diaphorase activity in other tissues (STRAUB, 1939; MAHLER *et al.* 1952; MAHLER, 1955; STRITTMATTER and VELICK, 1956), enzymes possessing diaphorase activity are readily extracted by mild procedures from brain homogenates. By the use of differential extraction, two enzyme systems have been obtained which catalyse the oxidation of reduced pyridine nucleotides by methylene blue, ferricyanide, 2:6-dichlorophenolindophenol, and vitamins K_1 and K_3. This communication reports on some aspects of the relationship of these enzymes to the brain particulate preparations from which they are obtained, their separation, and some of their properties.

MATERIALS AND METHODS

Chemical preparations. DPNH and TPNH were obtained from the Sigma Chemical Co. The concentration used was determined from the optical density at 340 mμ on the basis of the extinction coefficient, $\varepsilon = 6{\cdot}22 \times 10^3$ (HORECKER and KORNBERG, 1948). Menadione (2-methyl-1:4-naphthaquinone) was obtained from Merck, Sharp and Dohme, Inc., recrystallized from 50% ethanol, and fresh solutions were prepared each day by dissolving 8·6 mg in 2 ml of acetone and diluting the solution to 50 ml with water. Bovine serum albumin was obtained from Armour Pharmaceutical

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§ Senior Research Fellowship (SF-42) of the Public Health Service.

Co. Amylobarbitone (Amytal, sodium 5-ethyl-5-*iso*amylbarbiturate) was a gift from Gane and Ingram, Inc. Vitamin K_1 was a gift from Merck, Sharpe and Dohme, Inc., and was dissolved in a solution of Brij-35 according to WOSILAIT[1]. Methylene blue, 2:6-dichlorophenolindophenol, and potassium ferricyanide were commercial products. Flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) were obtained from the Sigma Chemical Co. The concentrations of FAD and FMN were determined from the optical density at 450 mμ by using as extinction coefficients $\varepsilon = 11{\cdot}3 \times 10^3$ and $12{\cdot}2 \times 10^3$ respectively (WHITBY, 1953).

Assay procedures. All assays were carried out at constant temperature, 28°–30°, in the Beckman DU or the Zeiss PMQ II Spectrophotometer, with cells of 1 cm light path. The rate of oxidation of DPNH or TPNH was measured by following the change in optical density at 340 mμ, readings being taken at 15 or 30 sec intervals. In experiments with menadione, because of the diphasic rate obtained with this acceptor, experiments were conducted with very low concentrations of both enzyme and menadione in order to ensure linearity (ENGLARD and STRECKER, 1956). With 2:6-dichlorophenolindophenol the rate of oxidation was determined from the change in optical density at 600 mμ by use of the extinction coefficient $\varepsilon = 20{\cdot}2 \times 10^3$ (STEYN-PARVE and BEINERT, 1958). With ferricyanide the rate of oxidation was determined from the change of optical density at 410 mμ by using the extinction coefficient $\varepsilon = 1{\cdot}0 \times 10^3$ (CRANE *et al.*, 1960). A unit of enzyme activity is defined as the amount of enzyme causing a change in absorption at 340 mμ of 0·001 per min with menadione as acceptor. Where total activities are recorded, they are calculated from the activity obtained with low enzyme concentrations in a range where proportionality is obtained between rate and enzyme concentration. Protein concentrations were determined by the method of LOWRY *et al.* (1951).

Enzyme preparations. The preparation used for the experiments with sonically disrupted particles has been previously described (GIUDITTA and STRECKER, 1959). The preparation of the soluble diaphorases follows. Beef brain was obtained from the slaughter house and was freed as much as possible from white matter, blood vessels, and connective tissue. The predominantly cortical tissue remaining was homogenized for 2 min with a Waring blendor in 10 parts of glass distilled water (w:v). The suspension was centrifuged in the cold for 45 min at 20,000 **g**. The sediment was re-extracted twice in a similar manner. The residue of the third water extraction was homogenized with 10 parts of 0·05 M-potassium phosphate buffer, pH 7·5 (w:v), and the centrifugation and re-extraction with phosphate were repeated twice. The experiments described below were conducted with the initial extracts obtained with water and phosphate.

RESULTS

We have previously reported on the pyridine nucleotide oxidizing activity of a particulate preparation obtained by subjecting a subcellular fraction rich in mitochondria to sonic vibration for short periods of time (GIUDITTA and STRECKER 1959). To obtain information about the enzymic components of this preparation, experiments were carried out with menadione which has been shown to react with several flavin enzymes (MAHLER, FAIRHURST and MACKLER, 1955; MAHLER, 1956). The increase in rate of DPNH oxidation determined by the addition of menadione was dependent on the concentration of DPNH used (Fig. 1). In the absence of menadione the concentration of DPNH required for maximal activity either in the presence or absence of cytochrome *c* was less than 1×10^{-5} M. This was determined from the observation that the rate of oxidation remained constant at least down to this concentration. In the presence of menadione the concentration of DPNH required for maximal activity was more than 1×10^{-4} M. The first points shown in Fig. 1 represent the lowest concentrations of DPNH where it was still convenient to measure initial rates with menadione present.

The effect of inhibitors on the oxidation of DPNH with menadione present was also studied. In preparations derived from other tissues, amylobarbitone is presumed

[1] We wish to thank Dr. W. WOSILAIT for the details of this method.

to act on a flavoproten preceding cytochrome *b* (CHANCE, 1956), antimycin before cytochrome *c* (POTTER and REIF, 1952), and cyanide on cytochrome oxidase (KEILIN, 1929; WARBURG, 1927). All three inhibitors depressed DPNH oxidation by the brain preparation. The stimulatory effect of menadione addition, however, was essentially the same in the presence of these inhibitors as in their absence (Table 1).

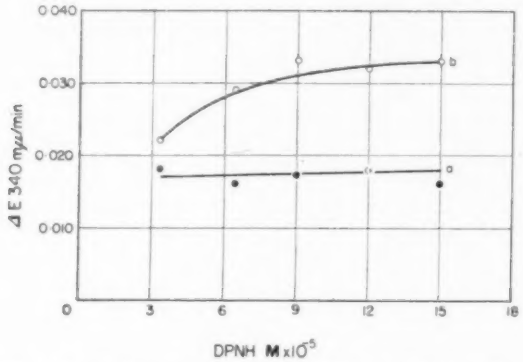

FIG. 1.—Effect of menadione on oxidation of DPNH. Incubation mixture contained 0·05 M-potassium phosphate buffer, pH 7·35 and 0·6 mg of enzyme protein. Total volume, 3·0 ml. Curve a. DPNH alone; concentrations as shown. Curve b. Same as curve a plus 0·4 μmoles of menadione.

TABLE 1.—EFFECT OF INHIBITORS AND MENADIONE ON DPNH OXIDATION
ΔE 340 mμ/min

Additions	Without menadione	With menadione	Difference
None	0·034	0·054	0·020
NaCN	0·002	0·030	0·028
Antimycin A	0·002	0·027	0·025
Amylobarbitone	0·001	0·021	0·020

The incubation mixture consisted of 0·05 M-potassium phosphate buffer, pH 7·31, and 0·28 μmoles of DPNH. In addition, as indicated, 0·4 μmoles of menadione, 1·2 μmoles of sodium cyanade, 0·55 μg of antimycin A, and 5·0 μmoles of amylobarbitone. Total volume 3·0 ml. 0·25 mg of enzyme protein was added to initiate the reaction.

Experiments with preparations subjected to various periods of sonic oscillation also indicated that the DPNH oxidizing activity in the presence of menadione differed in some respects from the other systems oxidizing DPNH which are present in these brain preparations. Exposure of the preparation to sonic oscillation for periods of time up to 5 min resulted in a decrease of DPNH oxidase activity of about 20 percent without any effect on the activity with menadione present (Table 2a). Increasing the time of sonic vibration beyond 5 min resulted in a progressive loss of DPNH oxidizing activity with or without menadione present. The loss of activity, however, was considerably greater in the system lacking menadione. Sonic oscillation for 30 min resulted in a loss of more than 90 percent of the units without menadione, and of less than 70 per cent of the units with menadione (Table 2b).

The preparation which was commonly used (5 min sonic treatment) was only

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slightly active with TPNH even in the presence of cytochrome *c*. The addition of menadione, however, caused an immediate and rapid oxidation of TPNH (Fig. 2).

In an attempt to bring into solution the enzymic systems that might be responsible for the data obtained, the particulate preparation was treated with 10 volumes of acetone at 0–3°, and the precipitate obtained washed with cold acetone and dried.

TABLE 2.—EFFECT OF INCREASING PERIODS OF SONIC OSCILLATION ON STABILITY OF DPNH OXIDIZING ACTIVITY

a			b		
Time (min)	Units/ml		Time (min)	Units/ml	
	−menadione	+menadione		−menadione	+menadione
1·25	1470	1550	5	1075	1160
2·5	1365	1800	10	696	920
3·75	1335	1590	15	369	865
5·0	1190	1575	20	192	620
			25	115	507
			30	58	373

The incubation mixture consisted of 0·05 M-potassium phosphate buffer, pH 7·3, 0·26 μmoles of DPNH, 0·4 μmoles of menadione in a total volume of 3·0 ml.

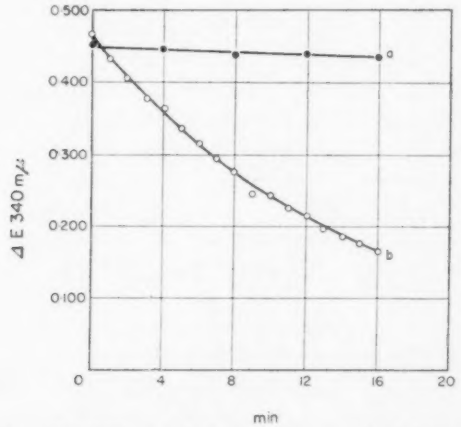

FIG. 2.—Oxidation of TPNH. Incubation mixture contained 0·05 M-potassium phosphate buffer, pH 7·35, 90 μmoles of EDTA (ethylenediaminetetraacetate), 0·52 mg of enzyme protein. Curve a. plus 0·22 μmoles of TPNH. Curve b. Same as curve a plus 0·4 μmoles of menadione.

The resulting powder was extracted with water. The aqueous extract was capable of oxidizing either DPNH or TPNH only when menadione was added. The data obtained from the studies conducted with this preparation are to be reported elsewhere (ENGLARD and STRECKER, unpublished observations.)

From the foregoing observations it appeared possible that a DPNH-menadione (and TPNH-menadione) system exists in brain particulate preparations which may be independent of other systems oxidizing DPNH. If this postulate is correct, this system has a different saturation level for DPNH than the other systems (Fig. 1), is

not affected by the inhibitors of the other systems (Table 1), is more stable to sonic oscillation (Table 2), and is soluble under the proper conditions. To test this hypothesis, experiments were conducted to determine whether or not an enzyme or enzymes whose properties included those postulated above might be extracted from brain. The extraction procedure is described in the Methods. With beef brain, the initial water extract contained most of the water extractable activity, and two further extractions with water only added about 3·5 per cent more units (Table 3). Since the

TABLE 3.—EXTRACTION OF DPNH-MENADIONE REDUCTASE ACTIVITY FROM BRAIN CORTEX

	Total units	% of Total
Original homogenate	63,770	100
First H_2O extract	30,000	47
Second H_2O extract	1,130	1·8
First phosphate extract	24,200	38·0
Second phosphate extract	2,940	4·6
Third phosphate extract	740	1·2
Final residue	1,920	3·0
Recovery	61,940	97·2

The incubation mixture contained 150 μmoles of potassium phosphate, pH 7·5, 0·3 μmoles DPNH 0·03 μmoles menadione, $0{\cdot}74 \times 10^{-6}$ M-FAD, and 1 mg bovine albumin in a total volume of 3·0 ml. In addition 1 μmole of KCN was present in the assay of the original homogenate and final residue.

residue still showed considerable activity, further extraction was carried out with dilute salt solutions. Our final procedure utilized 0·05 M-phosphate, pH 7·5, which almost quantitatively removed the remainder of the activity. The lack of activity in the third water extract, as compared with the high level of activity in the first phosphate extract, suggested that at least two different enzyme systems capable of reacting with menadione might be present in these homogenates. It was also possible, however, that the two extractable activities were the same enzyme bound to two different kinds of cell structures and that the portion bound to one kind of cell structure was more easily brought into solution. To compare the two activities, the enzyme extractable with water was purified about 200-fold, and some of the properties of this purified enzyme, the crude water extracted enzyme, and the crude phosphate extracted enzyme, were examined. For brevity, the water extractable enzyme will be referred to as enzyme 1 and the phosphate extractable enzyme as enzyme 2.

Pyridine nucleotide oxidation. The two enzymes were active with both DPNH and TPNH with menadione added. The optimum pH of activity of enzyme 1 with DPNH was 5·2–5·3; for TPNH a plateau in the region pH 5·7–6·7 was found. In the optimum pH ranges of enzyme 1 and at equal pyridine nucleotide concentrations the activity with DPNH was slightly greater than with TPNH. With enzyme 2 the pH optimum was 5·0–5·1 with both TPNH and DPNH. At relatively low concentrations of pyridine nucleotide (less than 1×10^{-4} M) the rate with TPNH was somewhat greater than with DPNH. The saturation level of DPNH, however, was considerably greater than with TPNH; as a consequence, at high concentrations of pyridine nucleotides (greater than 10^{-4} M) the rate with DPNH was greater than with TPNH. The K_m values (Table 4) for the two enzymes were obtained by a plot

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of the reciprocal initial velocity against the reciprocal substrate concentration (LINEWEAVER and BURK, 1934). Although the K_m values for the crude phosphate extract may be subject to revision when the enzyme is purified, as a comparison of half maximal rates of the two enzymes, the data are compatible with the non-identity of the two enzymes.

TABLE 4.—K_m FOR PYRIDINE NUCLEOTIDES FOR ENZYMES 1 AND 2

	Enzyme 1*	Enzyme 2
DPNH	$1{\cdot}2 \times 10^{-4}$ M	$5{\cdot}8 \times 10^{-4}$ M
TPNH	$1{\cdot}2 \times 10^{-5}$ M	$4{\cdot}5 \times 10^{-4}$ M

* purified.

The incubation mixture and experimental conditions were the same as described under Table 3. However, at high pyridine nucleotide concentrations the total volume was decreased to 1·5 ml and an insert providing a 1 mm light path was used. With enzyme 1, 0·1 μg of protein of specific activiy, 290,000 units/mg and for enzyme 2, 86 μg of protein of specific activity, 230 units/mg, were added to initiate the reactions.

Stimulation by flavin nucleotides. A number of soluble DPNH oxidizing systems have been reported in the literature to be flavoproteins (MAHLER, 1956). Whereas the first extracts of both enzymes were stimulated by both FAD and FMN (Table 5), the concentration of FMN required for maximal effect was about 10 times as high as that of FAD. This stimulation was observed with both DPNH and TPNH. Purification of the water-extracted enzyme resulted in an increased requirement for flavin nucleotides, a six- to seven-fold stimulation being obtained with the most purified preparations.

TABLE 5.—STIMULATION OF DPNH OXIDIZING ACTIVITY BY FLAVIN NUCLEOTIDES

	H_2O extract		Phosphate extract	
	units/ml		units/ml	
Additions	DPNH	TPNH	DPNH	TPNH
None	1700	1275	450	650
FAD	2250	2125	1000	950
FMN	2425	2125	950	825

The incubation mixture and conditions were the same as described under Table 3. The concentration of FMN was $1{\cdot}75 \times 10^{-5}$ M, and the concentration of FAD was $0{\cdot}74 \times 10^{-6}$ M. 12 μg of the water extracted protein and 43 μg of the phosphate extracted protein were used to initiate the reaction.

Reaction with other acceptors. In addition to menadione, activity of the initial extracts was also observed with Vitamin K_1, 2:6-dichlorophenolindophenol, ferricyanide, and methylene blue (Table 6). The activities of the two enzymes with each of these electron acceptors was determined at arbitrary concentrations of pyridine nucleotides and acceptors. These activities are not optimal (unpublished experiments) but are included here to give a first approximation of the relative activities with the various acceptors for each of the two enzymes taken separately. The concentrations

used represent convenient levels of initial optical density for spectrophotometric assay. Detailed kinetic studies are in progress.

Species distribution. Preliminary data have been obtained on the presence of the two enzyme systems in the brains of 4 mammalian species and of the pigeon. The activities extracted with water and phosphate differed markedly in each of the species examined (Table 7). With rabbit brain practically all of the activity present could be

TABLE 6.—ACTIVITY WITH VARIOUS ELECTRON ACCEPTORS

Acceptor	DPNH		TPNH	
	H_2O extract	Phosphate extract	H_2O extract	Phosphate extract
Menadione	100	100	100	100
2:6-Dichlorophenol-indophenol	75	59	49	45
Methylene blue	8	16	22	14
Ferricyanide	148	166	127	86
Vitamin K_1	8	17	24	11

The incubation mixture and experimental conditions were the same as described under Table 3. In addition, the concentrations of the electron acceptors were; 2:6-dichlorophenolindophenol, 0·110 μmoles/ml. methylene blue, 0·017 μmoles/ml; potassium ferricyanide, 0·69 μmoles/ml; Vitamin K_1, 0·14 μmoles/ml; 12 to 120 μg of water extractable protein and 43–108 μg of phosphate extracted protein were used to initiate the reactions. The activity with menadione is arbitrarily referred to as 100 per cent, and the activities with all acceptors are calculated in terms of DPNH oxidized.

TABLE 7.—DPNH-MENADIONE ACTIVITY IN THE BRAINS OF SEVERAL SPECIES

Species	Total units in homogenate/ g wet wt	Total extractable units	
		Extracted with H_2O	Extracted with Phosphate
Beef	16,470	8,990	7,590
Rat	7,100	6,460	350
Rabbit	2,560	2,330	0
Human	2,340	243	1,640
Pigeon	580	93	0

The incubation mixture and experimental conditions were the same as described under Table 3. Human brain was post mortem material removed several hours after death.

extracted with water, whereas with human brain the major portion of the activity required phosphate for extraction. The total activity present also varied greatly according to the animal. Beef brain contained approximately 28 times as much enzymic activity as pigeon brain per gram of tissue. Although a broader and more detailed investigation of species differences remains to be made, the data presented on the relative concentrations of water extractable and phosphate extractable units is compatible with the conclusion that the two enzyme systems are not the same.

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DISCUSSION

With the extraction of the two enzyme systems described in this report the number of possible alternate pathways in brain tissue increases to four for the oxidation of DPNH and to three for that of TPNH. Of these only one, the DPN-cytochrome system present in the mitochondria, appears to furnish a complete pathway for transfer of electrons from pyridine nucleotide to oxygen. Presumably two other particle systems present in the microsome fraction can also catalyse the reduction of cytochrome *c* by DPNH and TPNH (GIUDITTA and STRECKER, 1959). If, however, we can reason by analogy with the more extensively investigated tissues (STRITTMATTER and VELICK, 1956; MAHLER *et al.* 1958), it may be presumed that these microsomal systems do not contain either cytochrome *c* or cytochrome oxidase *in situ*, and therefore do not catalyse the oxidation of DPNH or TPNH by molecular oxygen via any known heme-containing system. In connection with this point it should be noted that transhydrogenase is present in brain in very low concentrations (KAPLAN, COLOWICK and NEUFELD, 1953).

The two enzymes described here fall into the general classification of diaphorases in view of the nature of the electron acceptors which can function, and are obtained by considerably milder methods than have been used for other animal diaphorases. However, the natural intracellular acceptor for these enzymes is not known. It is possible that the two enzymes are actually part of the normal respiratory chain of mitochondria which has been disrupted by the homogenizing or extraction procedures. Thus far we only have preliminary evidence dealing with this question. The supernatant solution of 0·25 M or 0·88 M-sucrose homogenates of brain contains enzyme 1. Similar findings for liver have led to the conclusion that the diaphorase present in the supernatant solution originated from the cytoplasm (STEIN and KAPLAN, 1958; ERNSTER and NAVAZIO, 1958). Furthermore, exhaustive extraction by both water and phosphate of rat brain cortex leaves relatively intact in the particulate residue the DPNH oxidase activity both sensitive and insensitive to antimycin A and amylobarbitone, namely the activity in the absence of any artificial electron acceptor.

Further purification and characterization of the biochemical and physical properties of these enzymes, now proceeding, may make it possible to elucidate the possible functions of and relationship to the alternate pathways of electron transport in the nervous system.

SUMMARY

A procedure is described for the isolation of two diaphorase systems from mammalian brain. One enzyme is extracted with water and the other with phosphate. The two systems catalyse the oxidation of either DPNH or TPNH provided suitable acceptors are added. The properties of the two systems in regard to the relative activities with both pyridine nucleotides and the effect of flavin nucleotides and various electron acceptors are compared. Some data are presented on the distribution of these enzymes in the brains of several species.

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THE ENCEPHALITIC ACTIVITY OF SOME EXTRACTIVES OF TISSUE FROM THE CENTRAL AND PERIPHERAL NERVOUS SYSTEMS*

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THERE is a considerable, and largely consistent, amount of data on the role of whole tissue from the central nervous system and of its extractives in the induction of experimental allergic encephalomyelitis (see reviews of ROIZIN and KOLB, 1959, and of WAKSMAN, 1959). On the other hand the information on the possible role of whole peripheral nerve in the induction of this disease is scanty and inconsistent. Thus, WAKSMAN and ADAMS (1956) found emulsions of whole peripheral nerve routinely pathogenic, whereas LUMSDEN (1949) reported a much lower incidence and ALVORD (1949), KABAT *et al.* (1948), and MORGAN (1947) all reported negative results. Extractives of peripheral nerve had not been assayed prior to this report. In most reports of experimental allergic encephalomyelitis, tissue from the peripheral nervous system has either not been examined or the examination has been quite cursory. Recently, however, WAKSMAN and ADAMS (1955, 1956) reported the consistent finding of damage to the peripheral nervous system and also noted some interesting species differences in this condition which they termed 'allergic neuritis'. They found that the rabbit and mouse behave similarly in that their central and peripheral nervous systems are both susceptible to Freund emulsions of central nervous system tissue but only their peripheral nervous system is susceptible to Freund emulsions of peripheral nerve tissue. Like those of the rabbit and mouse, the central and peripheral nervous systems of the guinea pig are both susceptible to Freund emulsions of central nervous system tissue, but the guinea pig is unique in that its central as well as its peripheral nervous system tissues are susceptible to Freund emulsions of peripheral nerve tissue (WAKSMAN and ADAMS, 1956).

An added complication is the presence of connective tissue in peripheral nerve. Whether or not this presents a specific immunological problem is not known, nor can one do more than conjecture concerning the possibility that such probably species specific tissue might explain some of the species specific susceptibilities mentioned above.

It was thought that the preparation and assay of some extractives of peripheral

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nerve might serve partially to elucidate some of these questions and, in addition, would supply further information on the chemistry of peripheral nerves.

METHODS

The original FOLCH-LEES proteolipid extraction technique (FOLCH *et al.*, 1951) was selected for two reasons: the presence or absence of encephalitogenic activity is well established for the various fractions, and the two most active fractions have quite different solubility characteristics. The guinea pig was selected as the test animal since only in this species has the production of both experimental allergic encephalomyelitis and allergic neuritis been reported to result from injections of emulsions based on peripheral nerve. As 60 g quantities of fresh tissues are required for the procedure as outlined by FOLCH *et al.* (1951), a large animal was necessary as a source of peripheral nerve. A local horse slaughter house kindly allowed the dissection necessary to secure such quantities of fresh tissues.* In addition to preparing extractives of peripheral nerve, it was also thought necessary to prepare similar extractives of bovine cerebral white matter, horse cerebral white matter, and horse spinal cord to provide some means of correlation with our earlier work and with reports in the literature. Other than the careful following of the FOLCH-LEES procedure we made no attempt further to characterize the extractives. Thus, while we will use the terms 'Proteolipid-A,' etc., we do not claim identity with similarly termed products prepared by other workers. We only state that the material we term 'Proteolipid-A,' for example, was separated at the stage in the FOLCH-LEES procedure where others have called the product Proteolipid-A.

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The relatively high connective tissue content of peripheral nerves and the lack of knowledge concerning extractives of peripheral nerve imposed a considerable dosage problem. Techniques that have been used in assaying both central nervous system tissue and its extractives and peripheral nerve have varied in the following ways: species of test animal, source of tissue tested, techniques of emulsifying the test tissue with adjuvants, total dosage of tissue per unit weight of test animal, ratio of tissue to adjuvants, and route of injection of emulsions. These differences render comparisons of reports at least hazardous. We arbitrarily decided to adjust the dosage on the basis of the amount of Proteolipid-A extractable from the amount of spinal cord that we had found optimal in our previous work. This amounted to approximately 1 mg. On this basis each test animal received an emulsion which contained 1 mg extractive regardless of the source or qualitative nature of the extract. In some cases extractions were done in duplicate, but extractives were not pooled, and all assays of encephalitogenic activity were done on the particular extractions listed in Table 1. The yields of extractives in these duplicate runs were usually within ± 10 per cent. The dosage used served to limit the variables to the site of central nervous system or peripheral nervous system from which the extract was obtained and the concentration of encephalitic activity in the particular extractive.

The emulsion consisted of equal parts of saline and Difco brand of complete Freund adjuvants plus 10 mg of extractive per ml of emulsion. Each animal received 0·1 ml of this emulsion† divided into four intradermal injection sites in the nape of the neck. Owing to the possible lability of extractives, no heat was used in making the emulsions.

The guinea pigs were of a mixed stain and weighed an average of 300 g. At least eight experimental and eight control animals were used in the assay of each extractive. The animals were housed four to a cage, two experimental and two controls. This seemingly large number of controls was necessary because we had previously found neurological disease in some of the guinea pigs locally available. The design was that if either or both controls showed any disease the entire cage was to be discarded. This did not prove necessary as no sign of neurological or other disease was found in any of the control animals. Controls received no injections, or injections of liver with adjuvants, or adjuvants with saline. The animals were weighed daily, examined for clinical neurological changes, and checked for symptoms of other, and perhaps transmissible, diseases that might involve the nervous system. Our routine method for localization of the disease process by means of electrolytic lesions was used (CLARK and BOGDANOVE, 1955). In addition to the lesion site, portions of the spinal cord and of each brachial plexus and of each sciatic nerve, with their connections with the spinal cord intact, were removed at autopsy and fixed in buffered formalin for histological study. The tissue was embedded in paraffin, sectioned, mounted serially, and stained with cresyl violet acetate (POWERS and CLARK, 1955). In some cases additional stains were made for peripheral nerve.

* We wish to thank Cherven's Industries, Inc., Elma, N.Y. for this privilege.

† Exact details of preparation of emulsion will be furnished on request.

RESULTS

Yields of extractives

The yields of both Proteolipid-A and the ether soluble fractions from the various central nervous system tissues were roughly comparable and in each case the ratio between Proteolipid-A and the ether soluble fraction yields was approximately 1 : 3 (Table 1). In contrast the yields of Proteolipid-A and of the ether soluble fraction from peripheral nerve were quite small, being in the case of Proteolipid-A about one

TABLE 1.—YIELD OF FOLCH-LEES PROTEOLIPID-A AND ETHER SOLUBLE FRACTION FROM BOVINE CEREBRAL WHITE MATTER, HORSE CEREBRAL WHITE MATTER, SPINAL CORD, AND PERIPHERAL NERVE

(60 g quantities of each tissue were used)

Type of extractive	Sources of extractives			
	Bovine cerebral white matter	Horse cerebral white matter	Horse spinal cord	Horse peripheral nerve
Proteolipid-A	1·056 g	1·098 g	1·358 g	0·187 g
Ether soluble fraction	3·257	3·002	4·461	0·963
Chloroform-methanol (2:1) insoluble residues	4·6	5·1	3·6	7·2

TABLE 2.—FRACTIONS OF SERIES SHOWING POSITIVE NEUROPATHOLOGY FOR AEM FOLLOWING INTRADERMAL INJECTION OF FREUND EMULSIONS OF PLA OR ESF FROM BOVINE CEREBRAL WHITE MATTER, HORSE CEREBRAL WHITE MATTER, SPINAL CORD, OR PERIPHERAL NERVE

Type of extractive	Sources of extractives			
	Bovine cerebral white matter	Horse cerebral white matter	Horse spinal cord	Horse peripheral nerve
Proteolipid-A	6/8	2/8	5/8	2/10
Ether soluble fraction	4/8	3/7	10/12	2/9

fifth that from the central nervous system and in the case of the ether soluble fraction about one third that from the central nervous system. As a result, the ratio between the yields of Proteolipid-A and the ether soluble fraction from peripheral nerve was about 1 : 5 rather than the 1 : 3 found in extractives from the central nervous system.

Encephalitic activity of extractives

The acute neurological symptoms of experimental allergic encephalomyelitis typical of guinea pigs receiving Freund emulsions of whole nervous tissue were absent from our series. Some of the animals showed weight losses or failure of normal growth but there were no paralyses, etc. Encephalitic activity of the extractives was assessed, therefore, solely on a histological basis (Table 2). In each group the number positive is shown as the numerator of a fraction and the number in the group as denominator. It is evident that all extractives were encephalitogenic for the central nervous system regardless of the source from which they were obtained. Differences

among groups are not statistically significant so no differences in potency of the various extractives can be derived from the data. There were only two cases in which definite pathologic changes were found outside the central nervous system. In one animal where Proteolipid-A from peripheral nerve had been injected there was a spinal ganglion infiltrated with round cells. The only other pathology observed was a mild cerebral meningitis. The second animal showing neuropathology outside the nervous system also had extensive involvement of the central nervous system following injection of a Freund emulsion of ether soluble fraction from horse spinal cord. The peripheral nerve involvement in this animal consisted of round cell involvement of a spinal nerve near the spinal cord, possibly including the nerve root. In two cases pathology was found outside the area of the electrolytic lesion whereas there was none in the immediate environs of the electrolytic lesion itself. These are the only two cases we have observed since we began using the 'electrolytic lesion' diagnostic technique (CLARK and BOGDANOVE, 1955). No pathology was found in the brachial plexuses or sciatic nerves of either experimental or control animals, neither was any pathology seen in tissues from the central nervous system or peripheral nervous system of any of the control animals.

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DISCUSSION

Since this study was begun* there have been three reports on the proteolipids of peripheral nerve. On the basis of a staining reaction, KOENIG (1959) has apparently demonstrated the presence of proteolipid in peripheral nerve. FINEAN, HAWTHORNE and PATTERSON (1957) have extracted horse sciatic nerve. In a series of five extractions they reported the astounding range of 0·54 per cent to 0·056 per cent proteolipid. Our finding of 0·31 per cent from the same source lies within the range reported by these workers. FOLCH and LEES (1959) incidentally reported a yield of 0·09 per cent from sciatic nerve (species not stated) which is again within the range reported by FINEAN *et al.* but considerably less than the amount we found. On the other hand, if KOENIG's (1959) staining reactions have quantitative implications, the yield from peripheral nerve should be much higher. For, since the stains he used are always heavier on the peripheral side of the glial-Schwann sheath junction, if the stained materials represent proteolipids in general, the yield from peripheral nerve should exceed that from the white matter of the central nervous system.

Our finding of a marked difference in the Proteolipid-A and ether soluble fractions of peripheral and central nervous system tissue are indicative of a probable unsuspected chemical difference in the myelin from these two sources. The nature of the extraction procedure is such that it is extremely unlikely that any systemic fat left clinging to the nerves could account for the relatively increased yield of the ether soluble fraction. Since the actual ratio between nervous and non-nervous tissue in the nerves we extracted is unknown it is problematical whether or not the small yields of proteolipid and the ether soluble fraction are due to either the small amounts of these extractives in peripheral nerve or to the large amount of non-nervous tissue. If the latter is the case, the data on extractives would be consistent with the findings of KOENIG (1959). Therefore, it will be important that future studies of the relative encephalitogenic potencies of peripheral and central nervous system tissue or their extractives must take into account the connective tissue content of peripheral nerve.

* A preliminary report on this work was presented. (1957) *Fed. proc.* **16,** 13.

A lesser activity of peripheral nerve could be anticipated solely on such a quantitative basis.

While several explanations for the variation in number of positive animals in the various groups could be advanced, the most logical would seem to be that the heterogeneous guinea pigs we used differed widely in susceptibility. It should be emphasized that the individual scores do not differ significantly nor do the combined Proteolipid-A scores differ from the ether soluble scores. However, scores of the groups in which extractives of peripheral nerve were used do differ significantly from the scores of those in which the extractives were from central nervous system tissue. Nevertheless, it seems best to emphasize only that extractives of peripheral nerve as well as those of central nervous system tissue are capable of inciting experimental allergic encephalomyelitis. There were only two questionable cases of allergic neuritis. In one of these, the extractive was from peripheral nerve and in the other from horse spinal cord. Compared to the results of WAKSMAN and ADAMS (1955, 1956) this is a surprisingly low incidence and may conceivably indicate that the active agent for allergic neuritis in the guinea pig may be destroyed by the extraction procedure we used. This needs further investigation.

Since dose-response curves for experimental allergic encephalomyelitis and allergic neuritis have not been computed by most workers in this field (including ourselves) it is not possible to state that failures of some workers to find allergic neuritis or experimental allergic encephalomyelitis following injections of Freund emulsions of peripheral nerve or central nervous system tissues were probably due to inadequate dosage. Such quantitative studies are needed. GOLDSTEIN *et al.* (1955) have also stressed this need for quantification in the field of experimental allergic diseases. Whether or not the low incidence of allergic neuritis which we found can be explained on the same basis, namely inadequate dosage, must also remain conjectural. We wish to emphasize only that both experimental allergic encephalomyelitis and allergic neuritis can be induced when either Proteolipid-A or ether soluble fraction is used, and further work is needed to establish the meaning of the many negative cases we have found.

SUMMARY

The ether soluble and the Proteolipid-A fractions of the FOLCH-LEES procedure have been prepared from horse peripheral nerve, horse spinal cord, horse cerebral white matter, and bovine cerebral white matter and tested for encephalitic activity. Total yields of extractives from the peripheral nerve tissues were approximately one third of those from the central nervous system sources extracted. The ratio between yields of the ether soluble fraction and Proteolipid-A from central nervous system is about 3 : 1 and from peripheral nerve 5 : 1. All extractives were encephalitogenic but the differences in activity were not statistically significant. The incidence of allergic neuritis was very low.

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THE EFFECT OF DI*ISO*PROPYLPHOSPHORO-FLUORIDATE (DFP) ON MOUSE BRAIN PHOSPHORUS METABOLISM

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THE pharmacological effects of di*iso*propylphosphorofluoridate (DFP)† are commonly attributed to its inhibition of nervous system cholinesterases. There are, however, a number of indications that other factors may be involved (MCNAMARA *et al.*, 1954).

Following inhibition by ^{32}P-labelled DFP, cholinesterase and other enzymes such as chymotrypsin are found on hydrolysis to yield [^{32}P]-phosphoserine; this suggests that the reaction of DFP with serine residues prevents the catalytic action of these enzymes (SCHAFFER *et al.*, 1953, 1954). The possibility that serine may also play an important role in enzymes of phosphorus metabolism is indicated by the relatively rapid rate at which protein-bound phosphoserine of animal tissues becomes labelled following the injection of ^{32}P-labelled orthophosphate (KENNEDY and SMITH, 1954).

On the basis of these considerations it seemed plausible that DFP might interfere with phosphorus metabolism by tying up serine groups which may be necessary for the functioning of the enzymes involved. Thus, KENNEDY and KOSHLAND (1957) have observed that serine may be involved in the action of phosphoglucomutase, and that this enzyme could be inhibited by DFP. Such an effect of DFP on the phosphorus metabolism of the nervous system could conceivably be responsible for some of the pharmacological effects of this agent.

A. STUDIES ON GROSS PHOSPHORUS FRACTIONS OF MOUSE BRAIN

Materials and Methods

Male mice, 10–15 weeks of age, from an inbred strain, were environmentally 'standardized' in individual cages for at least 1 week prior to the experiments (BARNUM, JARDETZKY and HALBERG, 1958). ^{32}P was obtained as orthophosphate from Oak Ridge and was diluted with 0·85% NaCl for injection. DFP was obtained in pure form through the courtesy of Dr. BERNARD JANDORF of the U.S. Army Chemical Warfare Service. It was diluted with 99% *iso*propanol to give stable stock solutions; just prior to injection a portion of such a stock solution was diluted with 0·85% NaCl to give the desired concentration.

The experimental animals received 4 μC of ^{32}P per g body weight intraperitoneally, immediately followed by subcutaneous DFP injection in a dosage of 2·5 mg per kg body weight. This dosage of DFP produced muscular fasciculation and incoordination in the mice but resulted in no fatalities in

* U.S. Public Health Service Pre- and Post-Doctoral Research Fellow of the National Institute of Mental Health, 1958–59. Material in this paper was taken from a thesis submitted to the Graduate School of the University of Minnesota in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

† The abbreviations used are: DFP, di*iso*propylphosphorofluoridate; ASP_1, acid-soluble inorganic phosphate; PrP, phosphoprotein phosphorus; RNA-P, ribonucleic acid phosphorus; PCh, phosphorylcholine; CDPCh, cytidine-diphosphate-choline; GPC, glycerylphosphorylcholine; CrP, creatine phosphate.

the course of these experiments. The control animals received the same dosage of ^{32}P and a subcutaneous injection of the appropriate volume of the vehicle used for DFP injection.

Exactly 3 hr after injection, each mouse was sacrificed by cervical dislocation, the brain was quickly removed, blotted, weighed, and homogenized in cold 0·85% NaCl. A portion of the homogenate was removed for subsequent cholinesterase assay by an electrotitrimetric method (GLICK, 1937) with acetylcholine chloride as substrate. The remaining homogenate was mixed with cold 30% trichloroacetic acid to give a final concentration of 10%. The acid-soluble and lipid fractions were prepared essentially according to the procedure of SCHNEIDER (1945). The ribonucleic acid fraction was prepared by extraction of the acid-insoluble, non-lipid tissue residue with hot 10% NaCl, precipitation of nucleic acid from the extract with ethanol, and hydrolysis with 0·3 N-NaOH, as described by DAVIDSON and SMELLIE (1952). Phosphoprotein phosphorus was liberated as orthophosphate by treatment of the remaining tissue residue with N-NaOH at 37° for 15 hr (SCHMIDT and THANNHAUSER, 1945).

The specific radioactivity (counts/min per μg P) of the acid-soluble inorganic phosphate (ASP_I) and of the phosphoprotein phosphorus (PrP) was determined essentially according to the procedure of ERNSTER, ZETTERSTROM and LINDBERG (1950). The *iso*butanol : benzene extracts were assayed directly for phosphorus content following $SnCl_2$ reduction, and for radioactivity with a dipping Geiger tube. The lipid and ribonucleic acid fractions were wet-ashed with H_2SO_4. The resulting solutions were assayed for radioactivity with a dipping Geiger tube, with the probable error of the count, corrected for background, being 5 per cent or less. Lipid phosphorus (Lipid-P) and ribonucleic acid phosphorus (RNA-P) were determined by the method of FISKE and SUBBAROW (1925).

In the experiment in which brain lactic acid and creatine phosphate were determined the materials and methods were the same as those described above except that the mice were sacrificed by immersion in liquid nitrogen, and their brains were removed while frozen, powdered, and directly homogenized in cold 10% TCA. Lactic acid was determined in the acid-soluble extract according to the method of BARKER and SUMMERSON (1941). The amount and specific radioactivity of creatine phosphate were determined essentially according to the procedure of ERNSTER *et al.* (1950).

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RESULTS AND DISCUSSION

The observed reduction in cholinesterase activity of the brain homogenate following DFP administration (Table 1) is probably greater than that actually existing in the brain prior to its homogenization (KEWITZ and NACHMANSOHN, 1957), but, nevertheless, it serves as an indication of the amount of DFP present in the brain. A reduction in specific radioactivity following DFP administration is quite apparent in the lipid and RNA fractions, the difference between the means for the control and DFP-injected groups being in both cases significant at $P < 0{\cdot}01$. On the other hand, the specific radioactivity of the Pr P fraction is probably not significantly reduced ($P > 0{\cdot}05$).

In order safely to conclude that the incorporation of ^{32}P into phospholipids and RNA had been reduced by DFP administration it would be necessary to know that the brain content of these substances was not increased. Quantitative data were obtained for the brain lipid-P content in the experiment described above; the mean values, plus or minus the standard error, for the control and DFP-injected groups were, respectively, $1{\cdot}95 \pm 0{\cdot}01$ and $1{\cdot}93 \pm 0{\cdot}01$ mg lipid-P per g wet wt of tissue. Recoveries of RNA-P and PrP were not quantitative; however, the amounts that were recovered did not differ significantly for the two groups. One can conclude, therefore, that incorporation of ^{32}P into brain phospholipids and RNA is reduced during the 3 hr period following DFP administration, whereas the incorporation into Pr P does not seem to be affected, at least not to a comparable extent. However, it is likely that the phosphoprotein fraction is of heterogeneous origin (AGREN, DEVERDIER and GLOMSET, 1954). Metabolically active components of this fraction may reach

isotopic equilibrium with ASP_i soon after the ^{32}P injection and could do so well within the 3-hr period even if their rate of formation were markedly reduced by DFP administration. Accordingly, one cannot definitely conclude, on the basis of the data above, that DFP administration does not affect incorporation of ^{32}P into some components of the phosphoprotein fraction.

The question arises as to whether or not the observed effect on ^{32}P incorporation into brain phospholipids and RNA could be ascribed to the existence of tissue hypoxia, since it is known that DFP can produce respiratory paralysis (HORTON *et al.*, 1946).

TABLE 1.—THE EFFECT OF DFP INJECTION ON THE 3-HR INCORPORATION OF ^{32}P INTO MOUSE BRAIN PHOSPHORUS FRACTIONS

Group	No. of animals	Cholinesterase activity*	Specific radioactivity*			
			ASP_i	Lipid P	RNA-P	PrP
Control	7	1·08 ± 0·04	85 ± 3	3·3 ± 0·1	3·5 ± 0·2	34 ± 1
DFP-Injected	8	0·14 ± 0·02	88 ± 3	2·4 ± 0·1	2·5 ± 0·1	32 ± 1

* The data are expressed as the group mean ± standard error of the mean; cholinesterase activity is expressed as m-equiv. acid released (from acetylcholine) per mg brain tissue per min × 10^5; specific radioactivity = counts/min/μg phosphorus.

If the mouse brain were hypoxic following DFP administration it should be possible to detect an increased lactic acid content and perhaps a decreased CrP content (GURDJIAN, STONE and WEBSTER, 1944). The data of Table 2 were obtained in an experiment identical in design to that described for Table 1 except that the mice were

TABLE 2.—THE EFFECT OF DFP ADMINISTRATION ON LACTIC ACID AND CREATINE PHOSPHATE CONTENT OF MOUSE BRAIN, AND ON THE 3-HR INCORPORATION OF ^{32}P INTO MOUSE BRAIN PHOSPHORUS FRACTIONS

Group	Lactic acid (μg/g)	Creatine-P (μg/g)	Specific radioactivity		
			Creatine-P	Lipid-P	RNA-P
Control (5 mice)	169 ± 7	158 ± 2	78 ± 6	3·3 ± 0·3	3·9 ± 0·3
DFP-Injected (5 mice)	125 ± 13	165 ± 2	82 ± 6	2·4 ± 0·1	2·9 ± 0·3

The data refer to group means ± the standard error of the mean.
Specific radioactivity = counts per min/microgram phosphorus

sacrificed by immersion in liquid nitrogen to preserve the brain levels of these two indicators of hypoxia (MCILWAIN, 1955).

The data on specific radioactivity of the lipid-P and RNA-P fractions confirm those of the previous experiment. Surprisingly, the apparent effect of DFP administration on brain lactic acid content ($P = 0{\cdot}02$) was the reverse of that expected if tissue hypoxia existed, whereas the CrP content or specific radioactivity were not significantly altered. The observed effect on brain phosphorus metabolism cannot therefore be ascribed to a primary tissue hypoxia, unless one proposes that the effect persists after an earlier, transient hypoxia.

A direct inhibition of the enzymes involved in the synthesis of brain phospholipids and RNA therefore remained a distinct possibility. However, these initial observations were rather crude, showing simply that there is a reduced isotope content in brain total lipid-P and RNA-P three hours after the administration of DFP. As REINER (1953) has cautioned, one cannot conclude on the basis of such information that the rate of formation of the compounds in question has been affected. Such a conclusion can only be rigorous when one has studied the relationship between the isotope content of a *single compound* and that of its *immediate metabolic precursor* at a *number of time points* following isotope administration, both in the presence and absence of the agent whose effect is under investigation.

An approach to such a study seemed feasible in the case of the lecithin class of phospholipids since recent work by KENNEDY and WEISS (1956) on liver and by MCMURRAY *et al.* (1957) on brain has clarified the path by which inorganic phosphate is incorporated into this phospholipid. A preliminary study revealed that the inhibiting effect of DFP administration on the incorporation of ^{32}P, observed in the case of total brain phospholipids, was also shown in the lecithin fraction. The incorporation of ^{32}P into lecithin involves a sequence of four reactions, the phosphorus-containing intermediates being, in order, adenosinetriphosphate (ATP), phosphorylcholine (PCh), and cytidine-diphosphate-choline (CDPCh). It seemed possible that the effect of DFP administration was on any one or several of these reactions, although the demonstration of the absence of change in the specific radioactivity of CrP made it unlikely that the incorporation into ATP was depressed. Attention was first given to the reaction in which the PCh moiety of CDPCh is incorporated into lecithin. An attempt to isolate CDPCh from mouse brain indicated that there was considerably less than one microgram of choline-bound phosphorus present as CDPCh per g. of brain. The prospect of losses during the several steps necessary for purification of this compound made this approach seem impractical. On the other hand, free PCh, which immediately precedes CDPCh as a precursor of lecithin, was reported to be present in brain to the extent of 10 μg of phosphorus per gram (DAWSON, 1955). Because its concentration is considerably higher than that of CDPCh it is very likely that isotopic equilibrium would exist between free PCh and the PCh moiety of CDPCh (ZILVERSMIT, ENTENMAN and FISHLER, 1943). One could, accordingly, treat specific radioactivity data on free PCh as though one were dealing with the immediate precursor of lecithin. A kinetic study was therefore made on the effect of DFP administration on ^{32}P transfer from free PCh to lecithin in mouse brain.

B. THE EFFECT OF DFP ADMINISTRATION ON THE TRANSFER OF ^{32}P FROM PHOSPHORYLCHOLINE TO LECITHIN

Materials and Methods

The description of the mice used, the ^{32}P dosage (4 μC/g), and the dosage of DFP (2·5 mg/kg) or of blank solution were the same as in the previous experiments. The mice were sacrificed by cervical dislocation at varying time intervals after the injections. Each brain was homogenized in cold chloroform : methanol, and the lipid extract was separated by centrifugation and washed essentially according to the procedure of FOLCH, LEES and SLOANE-STANLEY (1957). The 'washes' of the crude lipid extract were saved for the isolation of PCh (see below). The washed lipid extract was evaporated to dryness under reduced pressure, the lipid residue was hydrolysed with methanolic KOH, and phospholipid hydrolysis products were separated by paper chromatography with phenol : NH_3

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according to the method of DAWSON (1954). The phosphorus-containing lipid derivatives were located with an acid-molybdate spray (HANES and ISHERWOOD, 1949). The lecithin derivative, glycerylphosphorylcholine (GPC), which has the highest R_F and is clearly separated from the other compounds, was circumscribed with a rectangle of known area which was cut out and ashed directly with a $HClO_4$:H_2SO_4 mixture. To correct for the small amount of phosphorus in the paper (Whatman 1) itself, an identical area was cut from a blank lane of the same chromatogram and ashed in the same way. The final solutions were assayed for radioactivity with a dipping Geiger tube (probable error of assay less than 5%), and the phosphorus content was determined by the method of FISKE and SUBBAROW (1925).

Free PCh was present in the 'washes' of the crude lipid extract. To obtain sufficient PCh-P for a reliable assay, lipid washes from the lipid extracts of four mouse brains were pooled, adjusted to pH 10 with NH_3, and passed through a Dowex-1 (formate) anion-exchange column. After washing with distilled water, the adsorbed PCh was eluted with a small volume of 0·1 M-formic acid. The eluate was taken to dryness under reduced pressure at 37°. The resulting residue was chromatographed with phenol:NH_3; phosphorus-containing spots were located with acid-molybdate spray, cut out, ashed, and assayed for radioactivity and phosphorus in the manner described above. In this case, too, PCh has the highest R_F on the chromatogram and is well separated from trailing spots (DAWSON, 1955); the leading spot gives a positive test for choline esters (AUGUSTINSSON and GRAHN, 1953). Possible contamination of this PCh spot with free GPC, which has about the same R_F in the phenol:NH_3 solvent, was ruled out by the observation that GPC is not retained by Dowex-1. The PCh thus obtained was not separable from known PCh (obtained from California Corporation for Biochemical Research) on paper chromatography in two different solvent systems. An isotope-mixture technique was used for this demonstration: ^{32}P-labelled 'PCh', isolated from mouse brain as described above, was mixed with non-radioactive known PCh; the mixture was assayed for specific radioactivity and portions were chromatographed with both the phenol:NH_3 system and a propanol:NH_3 solvent (HANES and ISHERWOOD, 1949). The resulting single phosphorus-containing spot on each chromatogram was cut in half (in the direction of solvent flow), and the spot-halves were ashed and assayed for specific radioactivity. There was no significant difference between the specific radioactivity of the original mixture and that of any of the spot-halves. This suggests that the substance isolated from mouse brain was indeed PCh, since even a slight difference in R_F in either solvent system should have resulted in markedly different specific radioactivities of the leading and trailing spot-halves.

RESULTS AND DISCUSSION

The results (Table 3) were combined from two separate but identical experiments, each of which involved 24 mice divided into three groups, with four controls and four DFP-injected animals in each group. The interval between the injections (of

TABLE 3.—THE EFFECT OF DFP ADMINISTRATION ON THE SPECIFIC RADIOACTIVITY OF PHOSPHORYLCHOLINE PHOSPHORUS (PCh-P) AND LECITHIN PHOSPHORUS (GPC-P) IN MOUSE BRAIN AT VARYING TIME INTERVALS AFTER INJECTION

Group	Specific radioactivity*					
	1·5 Hr		3·0 Hr		4·5 Hr	
	PCh-P	GPC-P	PCh-P	GPC-P	PCh-P	GPC-P
Control	71 (70, 72)	0·82 ± 0·03	93 (90, 96)	2·61 ± 0·09	103 (101, 105)	4·35 ± 0·16
DFP-Injected	68 (63, 72)	0·53 ± 0·03	94 (94, 94)	1·23 ± 0·09	103 (99, 106)	2·08 ± 0·19

* Specific radioactivity = counts/min/μg phosphorus; Each PCh-P value is the average of the two values bracketed beneath it, each of which represents a pool from four brains. The eight individual GPC-P values at each time are summarized as their mean ± the standard error of the mean.

^{32}P and DFP or blank) and sacrifice was exactly 1·5 hr for the mice in one group, 3·0 hr for those in the second, and 4·5 hr for those of the third group.

These results clearly suggest that DFP administration reduces the incorporation of PCh into lecithin, since the specific radioactivity of PCh-P is apparently unaffected whereas the marked reduction in the specific radioactivity of GPC-P is significant at $P < 0{\cdot}01$ for all three time intervals.

These kinetic data allow one to estimate the 'turnover rate' of the brain lecithin 'pool', defined as the fraction of the lecithin pool which is newly formed per unit time, and how this is affected by DFP administration, provided that the following assumptions hold: (1) The specific radioactivity of free PCh-P equals that of the PCh-bound phosphorus present in free CDPCh, the immediate precursor of lecithin, at the time points considered. The likelihood of this being so was mentioned above. (2) The lecithin content of the brain is not affected by DFP administration. The method used for the isolation of lecithin does not allow quantitative recovery; however, the observation that total brain lipid phosphorus is not affected by DFP suggests that the lecithin content is probably also unchanged, since lecithin comprises a major portion of total brain phospholipid (MCILWAIN, 1955). Furthermore, the amount of brain lecithin phosphorus recovered (as GPC-P) with the method used was similar for the control and DFP-injected animals. (3) The lecithin molecule 'turns over' as a unit. If the PCh moiety is exchanged independently of the remainder of the molecule the calculated rate will represent only the rate of this exchange. (4) The entire brain lecithin pool is *metabolically* homogeneous. If this were not so, the calculated turnover rate would indicate the lower limit of the true value (JARDETZKY and BARNUM, 1958).

The first assumption reduces the two reactions under study to a single reaction of the form: $a \rightleftarrows b$, where a corresponds to phosphorylcholine phosphorus (PCh-P) and b to lecithin phosphorus (GPC-P). It is further assumed that the turnover rate of the brain lecithin pool is constant during the period under consideration, so that use can be made of the integral form of the ZILVERSMIT equation (ZILVERSMIT *et al.*, 1943): $\Delta B = K(\alpha - \beta)$, in which ΔB refers to the increment in specific radioactivity of the product, b, during the time period under consideration; K represents the turnover rate of the lecithin pool, while α and β refer to the areas, for that same time period, under the curves obtained by plotting the specific radioactivities of a and b, respectively, against time. Since the shapes of these curves prior to the 1·5 hr time point are not known, calculations are limited to the three hour period between the 1·5 and 4·5 hr time points. Such calculation of the normal (control) turnover rate yields the value 0·0133 as the fraction of the brain lecithin pool which is newly formed per hr. Following DFP administration the calculated value is reduced to 0·0057; i.e. only about 40 per cent of the control value.

Since the assumption was made that free PCh is in isotopic equilibrium with CDP-Ch, the implication is that DFP administration interferes with the reaction: CDPCh + Diglyceride → Lecithin + CMP. This could be due to a direct inhibition of the enzyme by DFP, as suggested at the outset of this paper. It could, however, also be due to a removal of necessary enzyme cofactors or to a reduction in the concentrations of diglyceride and CDPCh. For example, the inhibition of cholinesterase by DFP might be expected to lower the concentration of free choline and thus perhaps the concentration of PCh. This might, in turn, result in a reduced concentration of

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CDPCh and thus a reduced rate of lecithin formation. This could account for the data observed if there were relatively rapid isotopic equilibration between PCh and its precursors, as well as between PCh and CDPCh. Although the method used for the isolation of PCh was not quantitative, the amount recovered was consistently somewhat greater for the DFP-injected groups than for the control groups. This suggests that the above explanation is not correct and is the observation expected if the utilization, rather than the formation, of PCh is decreased.

On the other hand, inhibition of cholinesterase can be expected to result in an increased acetylcholine concentration which may be more directly responsible for the effect on lecithin formation. It is, of course, also possible that the effect is mediated by some agent elaborated outside the central nervous system.

The fact remains that one of the early consequences of DFP adminstration is an alteration of the normal pattern of brain phosphorus metabolism. WEBSTER (1954) reported that tri-*ortho*-cresylphosphate (TOCP), an organic phosphate anticholinesterase which, like DFP, produces nerve demyelination and paralysis in chickens, had no effect on the *in vivo* incorporation of ^{32}P into total phospholipids of the sciatic nerve of chickens. However, the effect was not studied until four days after the oral administration of TOCP so that earlier effects may have been lost. The ability of chicken sciatic nerve to incorporate ^{14}C-labelled acetate into total lipids *in vitro* was found to be depressed by prior administration of DFP (1 mg/kg subcutaneously) to the chicken (AUSTIN, 1957). This effect was most marked with nerves removed 24 hours after DFP injection, the earliest time point studied. The ability of DFP to produce demyelination and paralysis in chickens is probably not related to inhibition of cholinesterase (DAVIES and AUSTIN, 1953).

Such an early effect of DFP on brain lipid (and RNA) metabolism could very well be responsible for some of the early pharmacological effects observed. Thus, the majority of the effects of DFP administration to laboratory animals disappear within 24 hr whereas profound cholinesterase inhibition persists in the brain and elsewhere for long periods (KOELLE and GILMAN, 1946).

SUMMARY

The parenteral administration of di*iso*propylphosphorofluoridate (DFP) to mice resulted in a decreased incorporation of ^{32}P into total phospholipids and ribonucleic acid of the brain. Assay for brain lactic acid and creatine phosphate indicated that tissue hypoxia was not responsible for this effect.

A study of the kinetics of ^{32}P transfer from phosphorylcholine to lecithin indicated that DFP administration interfered with the final stages of lecithin formation.

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THE DIFFERENTIAL DIAGNOSIS OF NEUROLOGICAL DISEASES BY FRACTIONATING ELECTROPHORETICALLY THE CSF γ-GLOBULINS*

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ELECTROPHORETIC studies have shown qualitative and quantitative changes in the proteins of the cerebrospinal fluid in certain neurological conditions. Since the γ-globulins are almost always affected, the present report deals primarily with this fraction.

A specific abnormal distribution of the CSF γ-globulins has been reported by BOOIJ (1958) in cases of subacute sclerosing leucoencephalitis (SSLE). LOWENTHAL (1959) has also pointed out that in the common infectious types of encephalitis, some of the γ-globulin fractions migrate at a slower rate than in the normal CSF. It therefore appeared that, besides being usually increased in quantity, the γ-globulins might also show qualitative changes in diseases such as SSLE, multiple sclerosis, syphilis and African trypanosomiasis.

The electrophoretic pattern on agar gel of the CSF proteins in cases of multiple sclerosis differs from the normal in that there is an increase in the γ-globulin fraction, which is most striking in the region of slow migration. In SSLE, two γ-globulin subfractions migrate further towards the anode than in the normal, while in African trypanosomiasis the increase of the γ-globulins is more pronounced and more diffuse than in either multiple sclerosis or neurosyphilis, and without the presence of the slow fractions found in SSLE (Fig. 1).

These findings suggested that differentiation between multiple sclerosis, SSLE, neurosyphilis and African trypanosomiasis might be made on the basis of the electrophoretic pattern of the CSF proteins. Two methods were used to establish these different patterns:

(1) The determination of the rate of relative mobility (m_r) of the various fractions.

(2) The determination of the relative concentrations of each of the fractions by densitometry.

METHODS

The technique of agar gel electrophoresis has been previously described (KARCHER, VAN SANDE and LOWENTHAL, 1959). The relative mobility of the various fractions was computed by comparison with human albumin, siderophilin and depolymerized dextran (A.S.D. solution) run simultaneously. The distance between dextran and the line of maximum absorption of human albumin was taken as unity, according to WIEME's (1959) criteria (LOWENTHAL, KARCHER and VAN SANDE, 1959). Siderophilin was found at a distance of $0{\cdot}470 \pm 0{\cdot}013$ from dextran and 0·530 from albumin The β_1-globulin

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of human CSF migrates at the same rate as siderophilin while the rate of migration of the γ_3-globulin corresponded more nearly to that of dextran. The albumin migrates at the same rate as the control human albumin. The pre-albumins migrate toward the cathode at a greater rate than albumin. The γ_5-globulins migrate beyond dextran toward the anode and are conventionally said to have a negative mobility.

Extinction curves were drawn by scanning the plates every 0·25 mm at 546 mμ, by means of an Eppendorf spectrophotometer. The concentrations are then calculated as with paper electrophoresis by planimetry.

RESULTS

Results obtained for different rates of relative mobility. Electrophoretic patterns obtained after different periods of time were analysed in order to establish mean values and standard deviations for normal CSF which could be used as controls (Table 1).

In 84 cases, electrophoresis of CSF was run without the simultaneous electrophoresis of the A.S.D. pattern. The calculations were based on the assumption of perfect correspondence of CSF albumin with control human albumin and β_1-globulin with siderophilin. In each instance the value of $m_r = 1{\cdot}000$ was assigned to albumin and 0·470 to β_1-globulin (see Methods). In 37 cases and in a later set of 36 cases, samples of CSF were run concurrently with the A.S.D. solution (LOWENTHAL *et al.*, 1959). The relative rates of mobility in these sets enabled us to identify the various fractions.

Almost all CSF's were found to contain a pre-albumin, an albumin, α_1-, α_2-, β_1-, β_2- and γ_3-globulins. Other fractions such as γ_1- and γ_2-globulins appeared less frequently. At times intermediary or additional fractions were found. These were labelled α_1'-, α_3-, β_1'-, β_3-, γ_1'-, γ_2'-, γ_4-, γ_5- and γ_6-globulins.

TABLE 1.—RELATIVE RATES OF MOBILITY OF CSF PROTEINS ± S.D.

No. of cases	A.S.D. marker	Prealbumins			Albumin	Globulins				
						α_1-	α_1'-	α_2-	α_2'-	α_3-
84	without	—	1·261	—	1·000	0·870	—	0·680	—	—
			±0·014		—	±0·008	—	±0·013	—	—
37	with	—	1·262	—	1·005	0·878	—	0·682	—	—
			±0·024		±0·013	±0·013	—	±0·019	—	—
36	with	1·288	1·216	1·130	1·008	0·874	0·819	0·708	0·651	—
			±0·023		±0·008	±0·013	—	±0·016	±0·011	—

No. of cases	A.S.D. marker	Globulins								
		β_1-	β_2-	β_3-	γ_1-	γ_2-	γ_2'-	γ_3-	γ_4-	γ_5-
84	without	0·470	0·344	—	0·240	0·158	—	0·083	—	—
			±0·011	—	±0·017	±0·027	—	±0·013	—	—
37	with	0·472	0·348	—	0·225	0·142	—	0·055	—	—
		±0·028	±0·020	—	±0·016	±0·016	—	±0·022	—	—
36	with	0·481	0·364	—	0·244	0·165	0·103	0·070	0·026	0·032
		±0·012	±0·011	—	±0·015	±0·020	—	±0·018	—	—

The standard deviations have only been calculated for groups of 25 or more cases.
A.S.D. = Albumin, Siderophilin, depolymerized Dextran.

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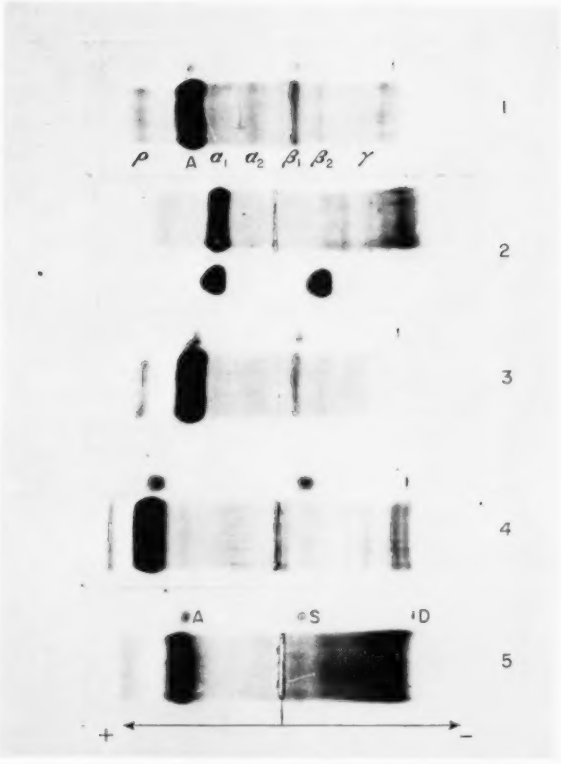

FIG. 1.—Agar gel micro-electrophoresis pattern of CSF from: 1 Multiple sclerosis. 2 Subacute sclerosing leucoencephalitis (SSLE). 3 Normal. 4 Neurosyphilis. 5 African trypanosomiasis.

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Because of the relatively rare appearance of some of those intermediary and additional fractions, the possibility that they may represent artefact or pathological response should be raised.

The appearance of so many different fractions in CSF would suggest the need for revising the accepted nomenclature. The problem of naming the new fractions should be studied. Until now we have extended the nomenclature developed from human serum but important variations found in studying the electrophoretic patterns of different species of insects and animals led us to designate the various fractions on the basis of their relative mobility (MISSELYN *et al.*, 1959). This system has been used to name the fractions found by electrophoretic examinations of the CSF from cases of multiple sclerosis, neurosyphilis, SSLE and African trypanosomiasis.

γ_4- and γ_5-globulins appeared in most cases of SSLE (Table 2), less frequently in multiple sclerosis and syphilis and hardly ever in cases of African trypanosomiasis. With regard to the last named, it should be mentioned that the electrophoretic run was obtained about one week after lumbar puncture performed in the Belgian Congo. All other fractions seemed to be present with the same frequency and with the same m_r as in normal CSF, with the possible exception of the γ-globulins.

TABLE 2.—FREQUENCY OF APPEARANCE OF DIFFERENT PROTEIN FRACTIONS (EXPRESSED IN %)

Diagnosis	Prealbumin	Albumin	Globulins								
			α_1-	α_2-	β_1-	β_2-	γ_1-	γ_2-	γ_3-	γ_4-	γ_5-
m_r	(1·216)	(1·008)	(0·874)	(0·708)	(0·481)	(0·364)	(0·244)	(0·165)	(0·070)	(0·026)	(−0·032)
Multiple sclerosis	100	100	80	100	100	100	80	90	100	—	60
Syphilis	100	100	100	100	100	100	60	80	90	50	—
SSLE	90	100	100	90	100	90	54	81	100	90	63
Trypanosomiasis	84	100	56	100	100	100	—	56	72	—	—

Fractions appearing in less than 50% of the cases are not mentioned.
m_r = relative mobility rates.

Results obtained by densitometry

The problems encountered in calculating normal control values for the concentrations of the proteins of the CSF are discussed below. CSF's from 53 cases were chosen as normal on the basis of their biochemical composition and the clinical picture of the case. The following mean values were obtained:

pre-albumin : 4·2%
albumin : 61·6%
α_1-globulin : 6·6%
α_2-globulin : 7·7%
β_1-globulin : 8·7%
β_2-globulin : 3·3%
γ-globulins : 5·1%

It will be noticed that the total for the above fractions is not 100%. This is due to the fact that additional fractions were found in some of the cases. These included the α_1'-globulins (4·6%) the α_3-globulins (4·5%) and the β_3-globulins (2·6%). They were not taken into account.

Among the γ-globulins, we are able to identify the following subfractions and to find their mean values:

γ_1- : 2·2%
γ_2- : 3·5%
γ_3- : 3·3%
γ_4- : 0·8%
γ_5- : 0·6%

Here the total exceeds the figure of 5·1% given as the mean for γ-globulins in normal CSF. This again is due to the fact that all the subfractions do not appear in all fluids.

Total γ-globulins were increased in all cases of diseases of the central nervous system under study (Table 3). In SSLE, the increae of γ_4- and γ_5-globulins accounts for the greater part of the increase. Although these fractions are slightly increased in multiple sclerosis and neurosyphilis, it is the γ_1-, γ_2- and γ_3-globulins which show the real augmentation. This is more marked in the latter disease. African trypanosomiasis gives a pattern which is very similar to that found in neurosyphilis (Table 4).

Therefore, although there is an abnormally high value for the γ-globulin fraction in all four of the diseases studied different subfractions are affected.

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TABLE 3.—CONCENTRATION OF γ-GLOBULINS IN THE DISEASES STUDIED

Diagnosis	Number of cases	Mean γ-Globulin concentration (% total protein concentration)	Mean (range)
Normal	53	5·1	(3–10)
Multiple sclerosis	10	11·1	(8·0–27·3)
Neurosyphilis	10	16·6	(5·0–36·0)
SSLE	11	25·4	(7·0–31·7)
Trypanosomiasis	14	29·2	(5·0–47·3)

DISCUSSION AND CONCLUSIONS

Increased γ-globulins in the CSF can be found in many disorders of the central nervous system and different subfractions can be shown to occur within the γ-globulins in different diseases. In addition to the clinical, electroencephalographic and anatomopathologic characteristics of SSLE, one might add the fact that the electrophoretic pattern of the CSF proteins shows increases in the γ_4- and γ_5-globulins. It is difficult to say whether this represents a specific reaction to the disease or is simply a reflection of its pathogenetic evolution. In chronic diseases such as multiple sclerosis, neurosyphilis and African trypanosomiasis these two subfractions are increased only minimally or not at all.

It would be interesting to compare our findings in SSLE with those obtained by electrophoresis of CSF in other types of subacute encephalitis, since it is conceivable that the increase in γ_4- and γ_5-globulins might turn out to be characteristic for this type of subacute illness. Although paper electrophoresis had already demonstrated some correlation between changes in serum and CSF, the use of electrophoresis in agar gel has revealed pathological changes which may help us to understand both the clinical disease and the pathological process.

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TABLE 4.—CONCENTRATIONS (MEAN) OF DIFFERENT PROTEIN FRACTIONS EXPRESSED AS PER CENT OF THE TOTAL PROTEIN CONCENTRATION

Diagnosis	Number of cases	Prealbumin	Albumin	Globulins								
				α_1-	α_2-	β_1-	β_2-	γ'_1-	γ'_2-	γ'_3-	γ'_4-	γ'_5-
m_r		(1·216)	(1·008)	(0·874)	(0·708)	(0·481)	(0·364)	(0·244)	(0·165)	(0·070)	(0·026)	(−0·032)
Normal	53	4·2	61·5	6·6	7·7	8·7	3·3	2·2	14·3	3·3	0·8	0·6
Multiple sclerosis	10	4·4	51·1	6·0	10·5	8·6	4·5	2·8	3·1	4·8	—	1·7
SSLE	11	3·6	50·5	3·3	4·5	7·4	5·8	3·7	4·5	6·2	10·6	3·8
Neurosyphilis	10	4·2	52·5	5·6	7·7	8·1	6·2	4·4	4·1	6·1	3·6	—
Trypanosomiasis	14	1·6	49·6	5·9	8·3	6·1	7·3	—	6·1	14·9	—	—

Fractions appearing in less than 50% of the cases are not mentioned. m_r = relative mobility rates.

Electrophoresis of the serum proteins showed little change from the normal and, like routine examination of the CSF, gave no specific indications of the nature of the condition in the neurological diseases that were studied.

SUMMARY

Agar gel electrophoresis of CSF, has demonstrated an increase of the normal components of the γ-globulin fraction (γ_1-, γ_2-, γ_3-) in multiple sclerosis, neurosyphilis and African trypanosomiasis. In SSLE, however, the additional subfractions of the γ-globulins migrate more slowly and have been designated γ_4- and γ_5-globulins.

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A CYTOPHYSIOLOGICAL STUDY OF THE FUNCTIONAL RELATIONSHIP BETWEEN OLIGODENDROGLIAL CELLS AND NERVE CELLS OF DEITERS' NUCLEUS

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PRESENT knowledge of the structure, metabolism and function of the neuroglia has been summed up in the proceedings of the Symposium on the Biology of Neuroglia (WINDLE, 1958). In spite of the fact that the neuroglial cells seem to outnumber the neurons of the cortex by a factor of at least 10, relevant data are scarce. This may be due in part to the lack of suitable methods for their analysis. The ordinary cytological treatment for structure analysis removes about 90 per cent of the organic material of the neuroglia, leaving relatively little to study. Furthermore, in our view, present methods for the preparation of glia by homogenization and differential centrifugation do not produce sufficiently pure fractions to be satisfactory.

The analysis of the neuroglia and the investigation of the possible functional relationship between the glia and the neurons become primarily a question of sampling. Technical advances in the preparation of extremely small and well defined samples of glial cells, and in the development of suitable methods for their analysis, has made possible a more direct approach. The experiments reported here are part of a series of studies of intraneuronal chemical changes under different functional conditions. The effects of physiological stimulation observable in the giant nerve cells of Deiters' nucleus, and in the glial cells which surround them, have been studied.

HISTORICAL REMARKS

At the beginning of this century, HOLMGREN (1899, 1901, 1904, 1914) made a series of careful *morphological observations* on the intimate relationship between neuroglial cells and nerve cells. He used mainly spinal ganglion cells from various species, and stained them in osmium tetroxide vapour. The cytologists of that time took more note of the osmophilic net-structure of the cytoplasm and HOLMGREN soon became entangled in a fruitless discussion about the similarity of his net-structure to that of GOLGI. The discussion of the glia cells in his papers was generally neglected and the problems relating to the function of the neuroglia were not pursued. HOLMGREN interpreted his pictures as showing that osmophilic granules were supplied to the nerve cell from the satellite cells through a system of delicate channels, a 'trophospongium', extending from these cells into the nerve cell body. He concluded that the glial cells had a nutritive function and therefore called them trophocytes.

In a high-resolution study, HESS (1958) has confirmed the main findings of HOLMGREN. The neuroglial cells of the cockroach formed a thin sheath consisting of several overlapping layers around the ganglion cells. The inner layer of the sheath penetrated into the nerve cell as delicate invaginations. The glial cytoplasm was, however, separated from the neuronal cytoplasm by 'double-layered membranes'. HESS (1958) has also stated that metachromatically-staining mucopolysaccharides, which he had earlier (HESS, 1953, 1955) found to characterize the intercellular spaces of the CNS, are in fact localized to the neuroglia.

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Contacts between oligodendrocytes and astrocytes, and the nerve cell including its processes can be seen in silver-stained sections. The same axon has contact both with the post-synaptic neuron and with its oligodendrocytes (SCHEIBEL and SCHEIBEL, 1955, 1958). In electronmicrographs the oligodendrocytes contain more particles and mitochondria than do the astrocytes (LUSE, 1956; FARQUHAR and HARTMANN, 1957; DEMPSEY and LUSE, 1958; and others). SCHULTZ *et al.* (1957) and DE ROBERTIS (1960) also stress the empty appearance of the astrocytes after fixation and embedding. The so-called neuropil is filled with the delicate, membrane-like processes from both the neurons and the neuroglia. The astrocytes ensheath the capillary walls and establish complicated contacts with the neurons and with the oligodendrocytes. The nerve cells are clearly isolated from the immediate blood supply by the glia. The only 'extra-cellular' space seems to be the 100–200 Å which separate the protoplasmic membranes.

In 1954, DE ROBERTIS and BENNETT described the existence of vesicles, 350–650 Å in diameter, in the endothelial cells of brain capillaries and in oligodendrocytes. They suggested that the vesicles were fluid globules, indicating a fluid transport by pinocytosis across the capillary wall. They also suggested that glial cells, Schwann cells and oligodendrocytes mediate the exchange between the nerve cell and the intercellular space.

WYCKOFF and YOUNG (1956, 1958), FARQUHAR and HARTMANN (1957) and SCHULTZ *et al.* (1957) have pointed out that morphological findings suggest that astrocytes may serve as the principal transport system to the nerve cells. DE ROBERTIS (1960) presents arguments for assuming that the astrocytes are the main centres for the salt exchanges in the brian, thus adding to the subject of the relationship between neuroglia and the blood-brain barrier. The narrow, 100–200 Å-wide clefts between the osmium-stained cell membranes offers another possible transport system. According to the present authors (see also HYDÉN, 1958, 1959) a cautious view ought to be taken as their dimensions *in vivo* may well be 300 Å instead of 100–200 Å. The fixation and embedding introduces, as a net result, a three-dimensional shrinkage, which is unlikely to affect all nervous structures in the same degree. Furthermore, the cytological procedure removes up to 80 per cent of the glial substance. SCHMITT (1959) has discussed the effect of the ionic strength, osmotic pressure and of the presence of metabolites on the equilibrium separation between membranes in a 100–200 Å channel. As SCHMITT has pointed out, certain indeterminacies characterize the electron microscopic approach to molecular biology.

In general terms, therefore, from a consideration of the morphological data, the astrocytes may be the principal transporting system from the capillaries to the oligodendrocytes and to the nerve cells. The intricate system of channels between all membranes, if it really exists, may also serve to transport solutes and metabolites. It may be, however, that the glial cells are the main factors in the blood-brain barrier. Based on tissue culture studies, LUMSDEN (1955, 1958) came to the conclusion that the ground substance in the CNS containing mucoproteins and mucopolysaccharides (HESS, 1953) is in fact glial cytoplasm. LUMSDEN is of the view that interstitial fluid channels are absent in the central nervous tissue. The transport of water and solutes would depend on diffusion through the cytoplasm of the neuroglial cells, and the blood-brain barrier would be, not a specific membrane, but the whole width of the gel-like glial matrix extending from the capillary endothelium to the neuron.

In recent years, the function of the oligodendrocytes has been mainly discussed in connection with myelination. In oligodendrocytes closely surrounding axons in young animals, LUSE (1956) and DE ROBERTIS *et al.* (1958) have described how concentric layers of membranes may be formed from vesicles, eventually building up the typical structure of myelin around the axons. According to this view, and contrary to the well-known theory of GEREN and SCHMITT (1954) and GEREN (1954), myelination in the CNS is a process of membrane synthesis within the oligodendrocytes.

In *tissue cultures*, both astrocytes and oligodendrocytes show rhythmic pulsatile activity (POMERAT, 1951, 1955; LUMSDEN and POMERAT, 1951). The tips of the thin, undulating membranes of the astrocytes showed uptake of fluid by pinocytosis (POMERAT *et al.*, 1957). If this pulsating activity occurred *in vivo*, the jelly-like, yet stable, substance of the neuroglia would form a biological substrate well suited for maintaining the homeostasis of the CNS.

With the exception of the oligodendroglia, the glia show in chemical studies a discrepancy between the results obtained with macro-chemical methods on glial tumours, or with histochemical methods on cerebral cortex, and the results obtained with cytochemical methods on isolated glial cells. HELLER and ELLIOTT (1955) found a low rate of O_2-utilization in gliomas. The oligodendrogliomas, however, had a higher oxidative mechanism than astrocytomas. ABOOD *et al.* (1952), working with glia cultures,

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found approximately the same cytochrome oxidase activity in glial cells as in nerve cells. POPE *et al.* (1956) studied the activity of respiratory enzymes in the different layers of the cortex. They concluded that the neuroglial cells did not make an important contribution to the high oxidative metabolism of the cortex. On the other hand the proteolytic activity of the glial cells was of the same order of magnitude as that of the neurons.

Working with isolated spinal ganglion cells, and their capsules containing satellite cells (oligodendroglia,) HYDÉN *et al.* (1958) found higher respiratory enzyme activity per unit volume in the glial cells than in the nerve cells. For cytochrome oxidase the relative activity of glial cells to ganglion cells was about 2, compared with 7 for succinoxidase. If the results of LOWRY *et al.* (1956), obtained on the same type of material, are recalculated on a volume basis, the same tendency is found. In the glial cells, the activity of phosphoglucoisomerase is 5 times higher than in the nerve cell per unit volume. That of glutamic-aspartic transaminase, 5 times and the activity of glutamic dehydrogenase 4 times higher in the glial cells than in the ganglion cells.

Working with Schwann cells, another type of glial cell, of the squid giant fibre COELHO *et al.* (1960) found the oxygen consumption to exceed that of the axoplasm. ROBERTS *et al.* (1958) estimated 10 enzymes in both the axoplasm and in the sheath of the squid fibre. They found the enzyme activities in the axon sheath lower in general than those in the axoplasm. They concluded that the sheath cells were more dependent on oxidative processes, particularly those of the citric cycle, than was the axoplasm.

Finally, interest has been shown lately in the capacity of glial cells both *in vivo* and in cultures, to respond *electrically* to direct stimuli. Slow, reversible, potential variations have been recorded by the micro-electrode technique (TASAKI and CHANG, 1958). There was a sudden depolarization followed by a slow return to the resting level. These potentials were of about one thousand times the duration of those recorded from nerve cells. CHANG and HILD (1959) found electrical stimulation to evoke a slow contraction in glial cells. Thus, like many other cells, the glial cells can produce bioelectrical potentials, which is not surprising. The character of these potentials as compared with those produced by the nerve cells may be relevant to the understanding of the functional relation between nerve cells and their associated glial cells.

EXPERIMENTAL

Preparation of extremely small samples of oligodendrocytes and of nerve cells

A prerequisite for a quantitative, microchemical study of glial cells, as well as of nerve cells, is a method for obtaining pure samples which are not too seriously damaged in the preparation. The tissues of the central nervous system are changed by most preparative methods. The delicate oligodendroglial membranes interlacing the corresponding processes from the astrocytes and from the neurons, are easily broken. We have chosen the direct and simple method of isolating the samples from the fresh tissue under the microscope. This technique has been successfully used for dissecting single, frozen and dried nerve cells by LOWRY and his associates (1953, 1954, 1957). For the present study, a somewhat different technique has been applied.

The animal was given an air embolus (20 ml of air injected in the ear vein). The animal was unconscious within a few seconds and its carotid arteries were cut to permit the remaining heartbeats to drain the blood from the body. The brain was rapidly removed, a slice was cut through the desired locus and placed in sterile 0·25 M-sucrose solution. From the nucleus to be studied were then removed single nerve cells, an approximately equal volume of the oligodendrocytes surrounding the nerve cell, and single nerve cells with an approximately equal volume of oligodendrocytes still adhering to them.

The cells, with their surrounding glial cells, were lifted out by hand under a stereomicroscope provided with hand-supports and at 64 or 100 times magnification. The instrument used was a 15, 18 or 28 μ thick, stainless steel thread, shaped as a spatula at its end with a point of a few μ.* The thread was inserted in a glass handle. This spatula was inserted under the nerve cell, and the cell plus adhering glial cells were lifted out of the thick section submerged in sucrose solution and placed in a small drop of sterile sucrose for dissection. By gentle manipulation the nerve cells were freed from the glial cells which clump together. Staining has shown that nerve cells completely free from surrounding glia can be obtained by this technique. It is important to note that most of the dendrites remain attached (Fig. 1), The enzyme activity in these parts of the cell is high. They also contain about 10 per cent of the ribose nuleic acids of the nerve cell.

* Stainless steel threads are supplied by A. B. Kanthal, Hallstahammar, Sweden.

The nerve cells are easily found if a drop of a highly diluted solution of methylene blue in isotonic sucrose is applied for some seconds to the desired locus. Note that the cells themselves must not be allowed to take up the stain. Only the synapses are stained, and they are seen as a finely dotted border around the area occupied by the unstained nerve cell. When experience has been gained in this type of work, no staining is needed for locating the nerve cells. In most instances, the appearance of the nerve cell in the incident light differs slightly from that of its surroundings.

One observation with the phase contrast microscope on such isolated nerve cells may be mentioned. The diameter of the nucleus was found to be much smaller (approximately 50 per cent) than that of nuclei from fixed and embedded cells. This can be explained by the fact that the usual fixation and embedding procedure removes 80–90 per cent of the organic material in the nucleus (HYDÉN, 1953; BRATTGÅRD and HYDÉN, 1954). The volume of the nucleus may then increase by the traction of the surrounding cytoplasm which has not lost material to this extent.

The glial cells removed from the nerve cell stick together and can be easily trimmed down to approximately the same volume as that of the nerve cell they surrounded. This was checked by forming the glial clump into a spheroid and determining the axes with a measuring ocular. Routinely, this was done by visual inspection only. The upper row in Fig. 1 shows three isolated nerve cells photographed in the phase contrast microscope. Below each cell is an equal volume of glial cells which originally surrounded that nerve cell. The amount of nerve threads or parts of dendrites left in the trimmed glial cells, found by teasing out and staining such material, is small and can probably be overlooked as a source of error. The isolation and dissection of 10 nerve cells did not take more than 5 min. The giant nerve cells of Deiters situated within the lateral vestibular nucleus of Deiters were used in the present investigation. For the description and nomenclature of this vestibular area, see BRODAL and POMPEIANO (1957, 1958) and POMPEIANO and BRODAL (1957). For short we have used the term "Deiters' nerve cells" to denote these giant nerve cells belonging to the lateral vestibular nucleus of Deiters. Using Golgi's rapid silver technique as described by SCHEIBEL and SCHEIBEL (1955) it was found that each large nerve cell is surrounded by 35–40 oligodendrocytes. The proportion of astrocytes did not appear to exceed 10 per cent.

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Stimulation of the vestibular nerve by rotation

Rabbits were placed in a 'tailor-made' box on a circular disc with the head away from the centre. The animals were rotated through 120° horizontally and 30° vertically with 30 turns per min and for 25 min per day for 1—7 days. The stimulation was sufficiently strong to subject the vestibular nerve to considerably increased demands, but slow enough not to cause any damage to the peripheral organ. This was checked by vestibular tests.

Material

In all, 181 rabbits were used for the present study; 27 rabbits for the determinations of the dry weight and the protein contents of the cells, 26 for the RNA and 128 for the enzyme determinations. A limited number of data were obtained from a large number of animals in order to avoid statistical errors depending upon the variation within the cells from each animal.

Methods of analysis (see Fig. 2)

Total organic mass. For the determination of the total organic mass of the fresh and dried samples in $\mu\mu$g per cell, X-ray microradiography at 8–10 Å was used according to the technique described by BRATTGÅRD and HYDÉN (1952, 1954) and HALLÉN and HYDÉN (1957). The mass of the cell can be divided into a protein fraction and a fraction of lipids soluble in chloroform.

The samples were placed on thin Al-foil in a special holder, dried in a high vacuum or precipitated with cold perchloric acid. An electronic cell analyser was used to get the integrated mass values of the samples. The instrument consists of a scanner dividing the sample into 12,000 parts, each of which may have an area of from 0·75 μ^2 to 6 μ^2. The mass values from each part are obtained by letting the signals pass an electronic function transformer. This transformer converts the non-linear transmission curve to a linear curve form, where every point is proportional to the mass (HYDÉN and LARSSON, 1956, 1959). The amplified and rectified potentials from each measuring area, which are proportional to the radiation intensity, are fed into the transformer, which acts as a voltage-dependent resistance. Ten diodes are connected and each of these is provided with a variable reistance. Each such unit can be set at a certain voltage. In this way, the function transformer can be calibrated against the reference

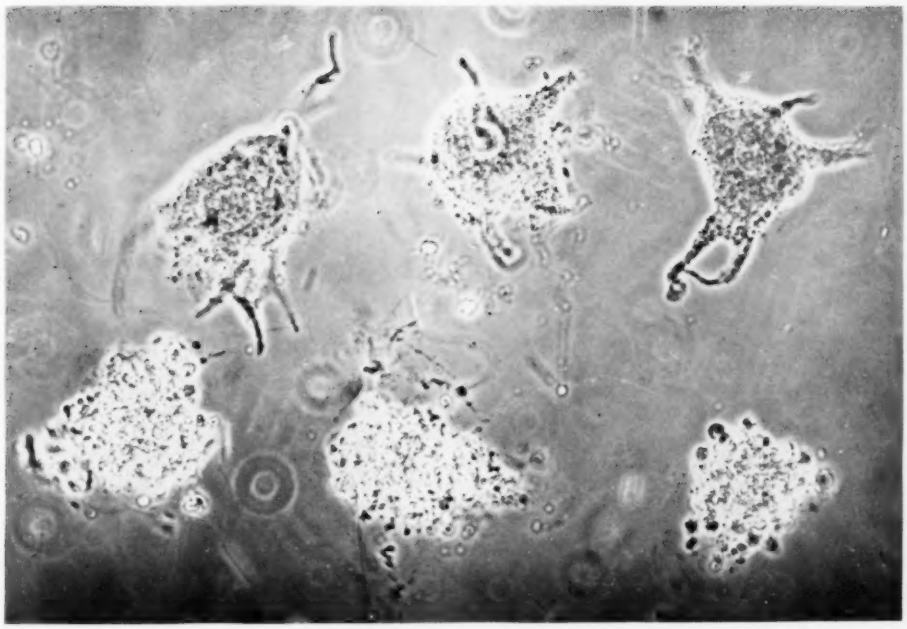

FIG. 1.—Upper row: three Deiters' nerve cells, dissected out free-hand, cleaned from the surrounding glia and photographed in the phase-contrast microscope. Lower row: three collections of glial cells each of which originally surrounded the nerve cell situated above. By free-hand dissection the glia samples were trimmed to approximately the same volume as that of the nerve cell. In the photograph they are pressed slightly against the glass and hence have a larger area than when ellipsoidal. ×170.

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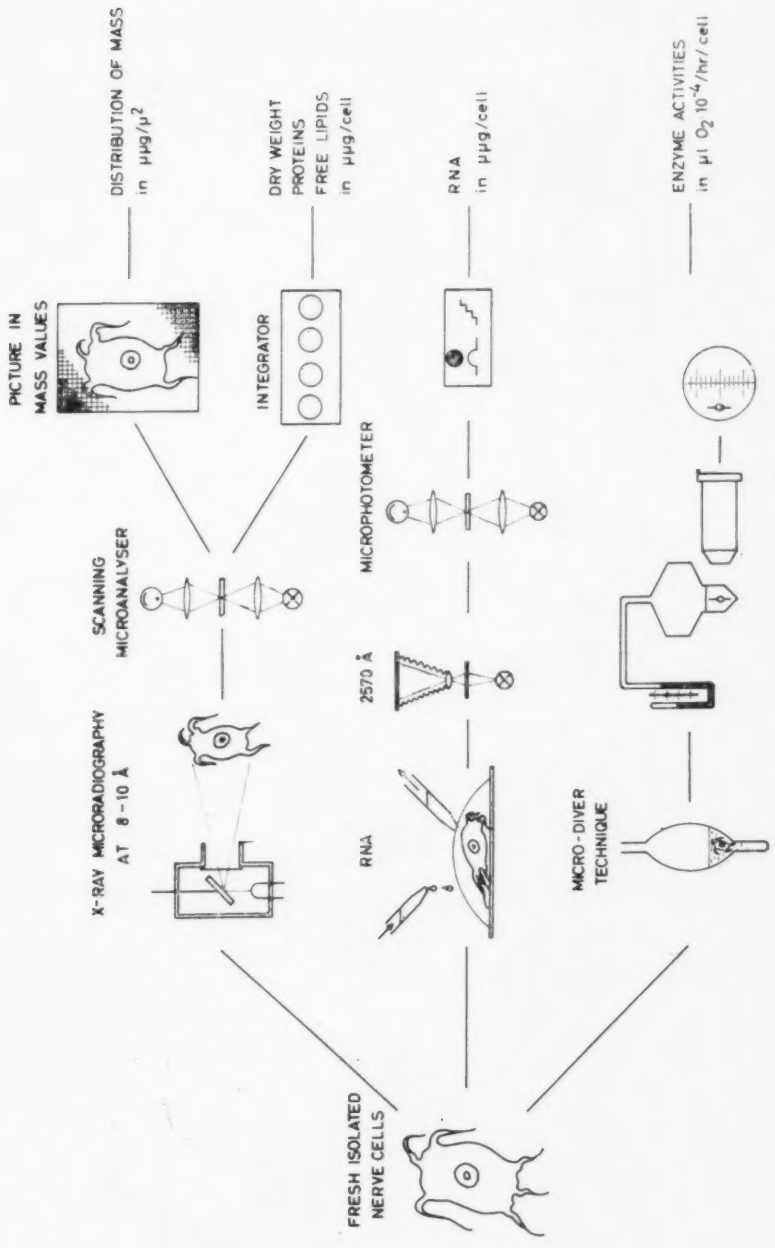

FIG. 2.—Survey of methods used in this tudy.

system of each X-ray preparation. The cell analyser determines 50 measuring areas per sec. In 4 minutes an integrated value of the mass of the whole cell can be obtained irrespective of its shape. In 40 minutes a picture in figures can be produced in which the distribution of the mass of the proteins is mapped as figures from 0 to 99. Information obtained in this way was used throughout most of this study.

The volume of the nerve cells including the main part of the dendrites can also be assessed by the X-ray method using the cell analyser. Several methods have been devised for the determination of the volume of fixed nerve cells or of those treated in other ways. Since fixation and histochemical treatment cause *inter alia* a high shrinkage volume, in neither this treatment nor the usual techniques for volume measurements are suitable for fresh cells. It has not yet been possible to adapt the interferometric technique for the volume measurement of whole fresh nerve cells. Therefore, the following method was used. The total dry weight of a nerve cell was computed by the cell analyser. The value for the dry weight per unit volume was obtained with the X-ray method using a frozen and dried section through the cell prepared in a cryostate (BRATTGÅRD and HYDÉN, 1954). A large number of values was taken by the computer to correct for errors due to variations in topographical thickness in the section. The volume of the fresh cell irrespective of its irregular form is then the total dry weight divided by the weight per μ^3 of the cell material. This assumption is justified as it has been shown that the organic mass is as high in the nerve cell nucleus as it is in the cytoplasm (BRATTGÅRD and HYDÉN, 1952; HYDÉN and LARSSON, 1956).

Determination of RNA. The determination of the amount of RNA per cell was carried out by the method described by EDSTRÖM (1953, 1958). The RNA is extracted with a buffered ribonuclease solution from each cell which is placed on a thin glass slide, precipitated with cold 1 N-$HClO_4$, and treated with ethanol and chloroform. The extract is optically homogeneous and the amount of RNA can be determined photometrically without the danger of errors due to tissue inhomogeneity. The extract is placed on a quartz glass and dissolved in a small volume of a glycerol-containing buffer under liquid paraffin. The amount of RNA per cell is determined photometrically by photographing the extracts together with a reference system at 2570 Å. The standard error is $\pm 5\%$ even for amounts down to 10–20 $\mu\mu$g of RNA.

Determinations of enzyme activities. These were carried out by ZEUTHEN's (1953) micro-diver technique at 36° and at 37°. Activities have been expressed as O_2 uptake in $\mu l \times 10^{-4}$/hr per nerve cell or per equal volume of glial cells. Zeuthen's apparatus requires a very high precision of temperature control. The water bath described below gave satisfactory results (Fig. 3).

The water bath consisted of a large container (about 60 × 60 × 70 cm) with walls of heavy glass plates fitted into a metal frame. In this was suspended a smaller container made of copper and with suitable glass windows which need not be water tight. The dimensions of the inner container were about 52 × 44 × 54 cm, so that the distance between the walls of the two containers was about 8 cm. This space and the inner container were filled with distilled water. A sensitive thermo-regulator and a heating element were fitted into the outer water space. The water in the outer container was stirred by a circulation pump and a motor-driven propeller which passed through a mercury lock in the bottom of the container. There was no heating in the inner container, but efficient stirring by a propeller which was driven by the same motor as the propeller in the outer container maintained an even temperature. The slight oscillations of the temperature due to the heater were damped by the large volume of water in the inner container. Both containers were covered with plastic plates leaving openings for the manometers. A cold light device was fitted into the inner container.

Preliminary experiments with the water bath were carried out in Krakow. They were made possible by the help of Mr. T. KOLKA from the State Mining School, Jaworzno, Poland, who assisted us in technical problems. The water bath was designed with Dr. S. LØVTRUP, the University of Göteborg, and was constructed by Mr. O. DICH, Copenhagen.

The manometers used in this work have been described previously (HYDÉN, LØVTRUP and PIGON, 1958). The final concentrations of substrates in the assay media are given (POTTER, 1957).

Cytochrome oxidase assay. 0·0375 M-Phosphate buffer, pH = 7·4; 10^{-4} M-cytochrome *c* (beef heart, Sigma); 0·0125 M-Na-ascorbate, prepared by neutralising ascorbic acid with NaOH just before the experiment; 5×10^{-4} M-$AlCl_3$; 1 mg/ml crystalline serum albumin.

Succinoxidase assay. 0·0375 M-Phosphate buffer, pH = 7·4; 10^{-4} M-cytochrome *c* (beef heart, Sigma); 0·025 M-Na-succinate; 5×10^{-4} M-$AlCl_3$; 5×10^{-4} M-$MgCl_2$; 1 mg/ml crystalline serum albumin.

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The addition of inert protein (LOWRY, ROBERTS and CHANG, 1956) lowers autoxidation and increases the reproducibility of the results.

Diver filling and measurement of enzymes. The method described by ZAJICEK and ZEUTHEN (1956) was used, but the divers were filled aerobically. A few points, which differ from the original procedure, are mentioned. These increased the reproducibility of the results. Dissected nerve cells, an equal

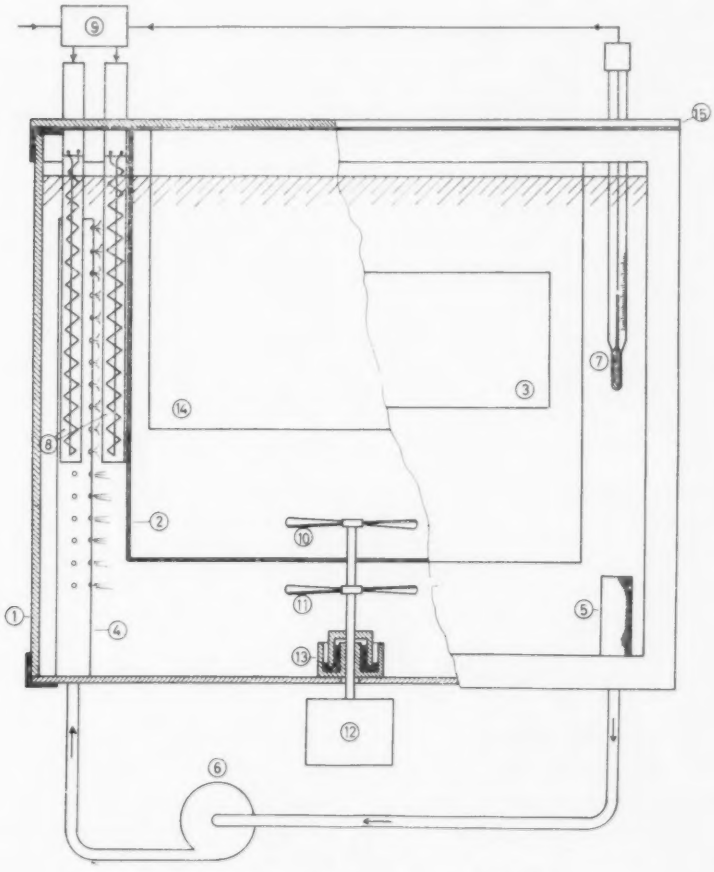

FIG. 3.—Schematic drawing of water bath used.

1. Outer container
2. Inner container
3. Window of the inner container
4. Inlet
5. Outler
6. Circulation pump
7. Contact thermometer
8. Heating element
9. Relays
10. Inner stirrer
11. Outer stirrer
12. Induction motor for stirring
13. Water lock
14. Cold light device
15. Plastic cover

volume of glial cells, or nerve cells together with an approximately equal volume of glial tissue were washed twice in sterile 0·25 M-sucrose, and transferred with a minimum amount of sucrose solution (not more than 0·5 μl) to a 75 μl drop of substrate in an agar-coated or a silicone-coated dish (Beckman 'Desicote'). All cells to be assayed were placed in the same substrate drop. The samples to be placed in the same diver were sucked into a constriction-braking pipette (HOLTER and LØVTRUP,

1949) with 0·2 μl substrate. The substrate with the cells (or cell) was sucked into the diver either directly from the constriction pipette, or after the pipette has been discharged on an agar-coated glass surface. In this way all divers could be charged with approximately the same volume of substrate. This was checked by subsequently weighing the divers (see below). The differences in the volume of substrate in different divers did not exceed 10 per cent. Correction for autoxidation was based on substrate volume (HYDÉN, LØVTRUP and PIGON, 1958).

The divers were made of Pyrex glass. No preconditioning in substrate was necessary. The divers' weights were 0·5–0·1 mg, which corresponds to a gas volume of 0·25–0·05 μl.

Four divers were run at the same time. For measurements of succinoxidase activity they were charged with 1, 2 or 3 nerve cells (or with the same number of nerve cells together with an equal volume of glial tissue), while the fourth diver contained substrate only (blank). For the assay of cytochrome oxidase activity, the divers were charged with 1, 2, 3 or 4 samples. No blank diver was run and the autoxidation was found by extrapolation. Recently, the experimental conditions have been slightly changed. A blank diver was taken at the beginning of the experiments and the autoxidation was directly determined. Not more than one sample was used in each diver. The flotation medium has been described previously (HYDÉN, LØVTRUP and PIGON, 1958).

After the measurements were completed, which generally took two hours (30 min for temperature equilibration and 90 min for the readings), the divers were placed on a filter paper in a closed container in which the air pressure could be lowered (suction by mouth). When this was done, the air bubbles in the divers expanded and forced the flotation medium out of the divers. The divers, containing now only substrate and cells, were weighed on a micro-analytical balance. They were dried for 24 hr at 100° and weighed again. From these weighings the volume of substrate in the divers was estimated. The calculations were carried out using ZEUTHEN's (1953) formula.

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RESULTS

The appearance of the fresh nerve cells in the phase constrast microscope

The Deiters' nerve cells belong to the largest cells in the central nervous system. The fresh cell body has the appearance of a moderately-filled paper bag with rounded off corners, from which the dendrites emerge, usually 6 to 8 from each cell. The nucleus appears spheroidal or spherical. The nucleus in these fresh cells is strikingly small in relation to the cell body. As already discussed, this may be due to the fact that the fixation and embedding procedure has not exerted its deleterious effect on the composition of these nerve cells.

If fresh nerve cells are placed in a highly diluted solution of methylene blue for ten seconds, the synaptic knobs on the surface of the cell body, on the dendrites, and even on the first part of the axon, will take up the stain and be seen as distinct, dark particles in the phase contrast microscope (Fig. 4a, b, c). The number of synaptic knobs is high both on the dendrites and on the cell body. We have found 15–20 end feet per 100 μ^2 as did WYCKOFF and YOUNG (1956). Assuming a total surface area for the cell + main part of the dendrites of 50,000 μ^2 this would give approximately 10,000 synaptic knobs per cell, a figure higher than that given by WYCKOFF and YOUNG. This difference is due to the fact that we have included the dendrite surface. The area of the latter was estimated to be 31,000 μ^2. Even the beginning of the axon has a small number of synaptic knobs. The assumption that the structures which took up the methylene blue and which were made clearly visible in the phase contrast microscope were in fact synaptic end feet, was confirmed by silver staining on isolated cells.

When the methylene blue solution was allowed to act for several minutes, the whole nerve cell gradually became stained, in the order, nucleus, nucleolus, cytoplasm. The cytoplasm showed the typical Nissl pattern when the optically denser

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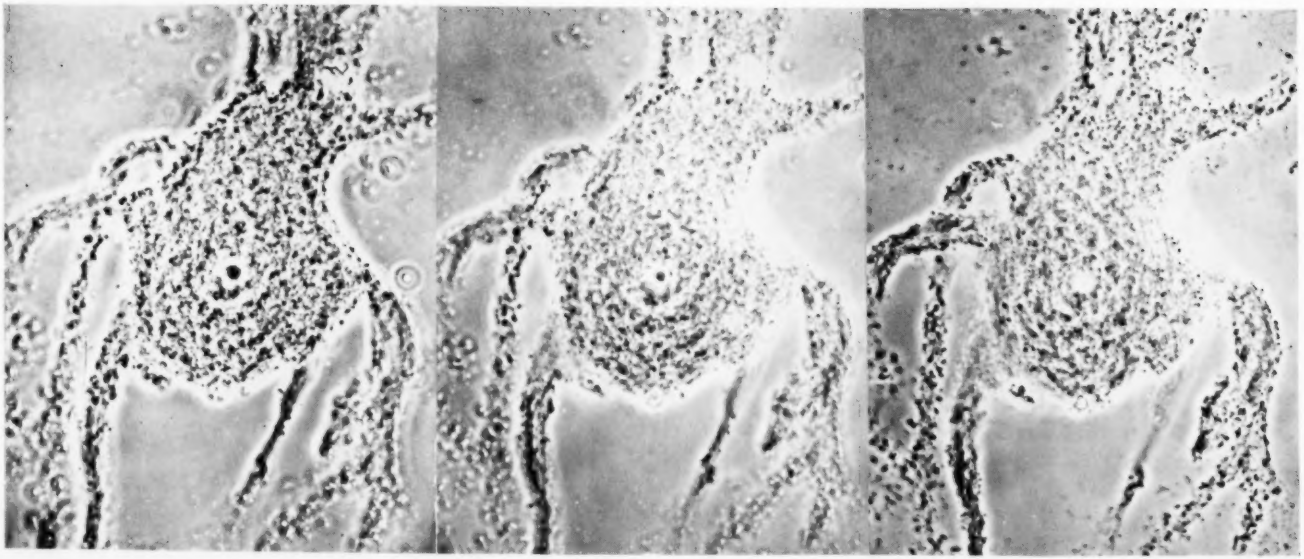

(a) (b) (c)

FIG. 4.—Fresh and cleaned Deiters' nerve cell photographed in the phase-contrast microscope at three different focusing levels. The small dense particles on the cell body and dendrite surfaces represent synaptic knobs which have been stained with a highly diluted methylene blue solution. Note the density of knobs in the photograph to the left. ×750.

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parts observable in the phase contrast microscope had taken the stain. This phenomenon has been observed in tissue culture preparations by DEITCH and MURRAY (1956).

For the determination of enzyme activities no cells with stained nuclei were used.

The dry mass of fresh Deiters' nerve cells and oligodendroglial cells

The distribution of organic substance within these large nerve cells is of interest. Analyses were made using the computing cell analyser on 5 μ sections through fresh cells which had been frozen and cut in a cryostat and dried (HYDÉN and LARSSON, 1956). The density (mass per unit volume) of the nucleus was 0·23 $\mu\mu g/\mu^3$ which is approximately that of the cytoplasm and of the main part of the dendrites. Differences were found in the density of the cytoplasm, which decreased successively from the region of the nuclear membrane towards the periphery of the cell, where the density was 25% less than that near the nucleus.

In a typical Deiters' nerve cell with a volume of 93 200 μ^3, the total dry weight of the nucleus was computed as 800 $\mu\mu g$, and of the nucleolus as 100 $\mu\mu g$, since the density of the latter was found to be 0·38 $\mu\mu g/\mu^3$. The dry weight of the cytoplasm was determined as 20,800 $\mu\mu g$ (HYDÉN, 1959). The proteins of the nucleus amounted to 650 $\mu\mu g$ and those of the cytoplasm to 16,600 $\mu\mu g$.

Vestibular stimulation

Vestibular stimulation produced a significant increase in the dry weight of the organic material of the large nerve cells, including the weight of proteins per cell (Table 1). The mean weight of the nerve cell was 20,094 $\mu\mu g$ for the control and 22,570 $\mu\mu g$ for the experimental, with approximately the same variation coefficient.

TABLE 1.—THE EFFECT OF STIMULATION ON DEITERS' NERVE CELLS FROM THE RABBIT

Dry weight and weight of protein ($\mu\mu g$/cell), from controls and from animals stimulated 25 min/day for 7 days. In all 22 animals were used.

	N	Dry weight (mean)	V	Weight of protein (mean)	*P*
Stimulated	64	22570	28	18060	0·01**
Controls	64	20094	23	16080	

N = number of cells analysed
V = the coefficient of variation, $\frac{s \times 100}{\text{mean}}$
P = probability after *t*-test

The weight of fresh, frozen and dried oligodendrocytes per unit volume was 0·20 $\mu\mu g/\mu^3$, i.e. approximately the same as that of the nerve cells they surround. Hence, it was possible to compare directly the enzyme activity of the nerve cell and glial cells. It may be noted that 70–80 per cent of the glial cells consist of lipids extractable with chloroform, compared with only 20 per cent extractable lipids of the nerve cells. This finding strengthens the conclusion drawn below of the enzyme activity of the glia as compared with that of their nerve cells.

The volume of Deiters' nerve cells of control and experimental animals

In view of the observation that regenerating nerve cells (BRATTGÅRD *et al.*, 1958) increased in volume by 150 per cent during the outgrowth period, owing to uptake of water, and during the maturation period owing to nucleoprotein increase, it seemed of interest to investigate possible volume changes in the present study. The mean volume of the control nerve cells was 88,000 μ^3, the dry weight per μ^3 being 0·23 $\mu\mu$g. The dry weight per μ^3 of the experimental cells was 0·26 and hence the mean volume was 87,000 μ^3. Thus no real volume change was detected.

The RNA of Deiters' nerve cells and their oligodendroglial cells

It has not yet been possible to determine directly the total amount of RNA in the nucleus of the Deiters' nerve cells. It has been estimated to be at least 20 $\mu\mu$g, but not more than 40 $\mu\mu$g. Furthermore, previous results have shown that the amount of nuclear RNA may vary, e.g. with the physiological condition or after virus infection or damage (HYDÉN, 1943, 1947; HOCHBERG and HYDÉN, 1949). The RNA content of the nucleolus is 1·5 per cent (W/v). The cytoplasm contains the largest amount of RNA, approximately 1·500 $\mu\mu$g or, if calculated in terms of the dry protein, 9 per cent RNA. Among the few cells which can compete with the large nerve cells as

TABLE 2.—THE EFFECT OF STIMULATION ON THE RNA OF DEITERS' NERVE CELLS AND GLIAL CELLS

RNA content ($\mu\mu$g/cell) from controls and from rabbits stimulated 25 min/day for 7 days. In all 26 animals were used. Notation as in Table 1.

		N	RNA content (mean)	V	*P*
Deiters' nerve cell	Stimulated	105	1612	11	0·01**
	Controls	114	1545	12	
Glial cells	Stimulated	30	85	22	0·001***
	Controls	25	123	35	

N = number of cells analysed
V = the coefficient of variation, $\frac{s \times 100}{\text{mean}}$
P = probability after *t*-test

RNA-producers are the pancreatic exocrine cells from small animals with a high metabolic rate.

Altogether 500 RNA determinations were performed. The data presented in Table 2 show that the vestibular stimulation caused a significant increase of the RNA per nerve cell, but a decrease of the RNA per equal volume of the oligodendrocytes.

In spite of the fact that glial cells lack Nissl substance—the chemical substrate of which is lipo-ribonucleoproteins—they nevertheless contain RNA in amounts which cannot be overlooked. The finding of a RNA content as high as 120 $\mu\mu$g in this exceedingly small volume of glial cells, should be noted by neurochemists. Since the glial cells outnumber the nerve cells by a factor which is probably greater than 10, it follows that a RNA value obtained from a piece of 'grey tissue', even if it is small, represents the nucleic acid content of the glial cells rather than that of the nerve cells.

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The enzymic activities of Deiters' nerve cells and their oligodendroglial cells

The data in Table 3 show that in the control samples, both the cytochrome oxidase and the succinoxidase activity are considerably higher in the oligodendrocytes than in the nerve cells, determined per unit volume. Vestibular stimulation brought about a reversal of this situation. The respiratory enzyme activity increased significantly in

TABLE 3.—THE ENZYME ACTIVITY OF DEITERS' NERVE CELLS AND GLIAL CELLS FROM RESTING AND STIMULATED ANIMALS

Enzymic activities expressed as $\mu l\ O_2 \times 10^{-4}$/hr per nerve cell or per same volume of glial cells surrounding the nerve cell from controls and from animals stimulated 25 min/day for 7 days. In all 78 rabbits were used.

Nerve cell		Cytochrome oxidase					Succinoxidase				
		N	*n*	Mean activity	V^s	*P*	*N*	*n*	Mean activity	V^s	*P*
	Controls	34	16	4·2	58	0·02*	37	17	2·2	60	0·001***
	Stimulated	40	19	6·7	51		57	23	6·0	46	
Glial cells	Controls	34	17	11·5	30	0·001***	30	13	4·5	46	0·6
	Stimulated	35	17	3·0	36		40	23	4·0	56	

V^s = the coefficient of variation $\frac{s \times 100}{\text{mean}}$

N = number of cells analysed
n = number of measurements
P = probability after *t*-test

the nerve cells, but decreased in the glial cells. The decrease was significant for the cytochrome oxidase.

Simultaneous analyses were carried out on 150 samples consisting of a nerve cell together with an equal volume of glial cells, determining one, two or three samples in one micro-diver. The values were lower than expected: in the controls, 11·1 for cytochrome oxidase (expected 15·7), 4·8 for succinoxidase (expected 6·7). In the stimulated samples, 8·1 for cytochrome oxidase (expected 9·7) and 6·2 for succinoxidase (expected 10·0). There may be several explanations for this result. The most probable seems to be that the number of glial cells was too small. It is more difficult to judge volume of glial cells when they remain as a layer around the irregular nerve cell. When three samples are used in one micro-diver, there may also be an insufficient diffusion of O_2, and inhibition may result.

Effect of vestibular stimulation for 25 minutes

Table 4 shows the effects of vestibular stimulation for only 25 min on the Deiters' cells and their oligodendroglia. The animals were killed one hour after the experiment. No significant effect was observed, except in the dry weight/cell. The intensity of the vestibular stimulation and the time required to produce changes in enzymic activities as profound as those observed after stimulation for 7 days remain to be investigated.

TABLE 4.—DRY WEIGHT OF NERVE CELLS FROM DEITERS' NUCLEIC OF THE RABBIT

Dry weight ($\mu\mu$g/cell) from controls and from animals stimulated for 25 minutes only. In all 5 rabbits were used. Notation as in Table 1.

	N	Dry weight (mean)	V	*P*
Stimulated	22	22940	29	0·01**
Controls	64	20094	23	

TABLE 5.—ENZYMIC ACTIVITIES OF DEITERS' NERVE CELLS AND GLIAL CELLS FROM DEITER'S NUCLEUS

Enzymic activities expressed as μl $O_2 \times 10^{-4}$/hr/sample from controls and from animals stimulated for 25 min only. In all 50 rabbits were used.

		Cytochrome oxidase			Succinoxidase		
		n	Mean activity	*P*	*n*	Mean activity	*P*
Nerve cells	Controls	18	4·2	0·3	17	2·2	0·4
	Stimulated	13	3·4		18	1·8	
Glial cells	Controls	17	11·5	0·05*	15	4·5	0·4
	Stimulated	13	9·0		13	3·9	

n = number of measurements
P = probability after *t*-test

DISCUSSION

RNA and protein production

With values up to 1550 $\mu\mu$g of RNA per cell, the large nerve cells seem to be outstanding among somatic cells as RNA-producers. The turnover of both RNA and proteins in the neurons is likewise high. For a discussion of these problems see the survey by Hydén (1960). It seems reasonable to conclude, on the basis of data available, that the large neuron is an enormous gland like structure, fulfilling its function as a steady and rapid producer of RNA, proteins and lipids.

As a background to the discussion of the results presented in this study, some comments on the status of the RNA in the nerve cell may be justified. The Nissl substance consists of liporibonucleoproteins. In the cytologically fixed cell, however, all of the lipid and much of the protein are extracted, leaving the ribonucleic acids as the stainable substance. In electron micrographs of fixed tissue the Nissl substance appears to be composed of membrane-like structures carrying minute particles (PALAY and PALADE, 1955; SJÖSTRAND, 1956).

The physico-chemical state of the RNA within the nerve-cell body can undoubtedly change considerably, as has been shown by analyses of chromatolytic nerve cells undergoing retrograde reaction during regeneration (BRATTGÅRD *et al.*, 1958). The chromatolytic cells during the rapid outgrowth of the axon had the same amount of

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RNA as had the control cells. In UV-micrograph at 2570 Å*, the RNA aggregates showed, however, a finely dispersed appearance quite different from the highly absorbing aggregates characterizing the nucleoproteins of the control nerve cells. Furthermore, the incorporation of ^{14}C-labelled precursors into the finely dispersed RNA of the chromatolytic cells was double that of control nerve cells (BRATTGÅRD *et al.*, 1958). Therefore, it was assumed that the changed RNA in the chromatolytic cells signified a more active status of the nucleic acids. Thus, not only can the production of RNA in nerve cells increase following functional demands, but the RNA (or some part thereof) can change from an active to a more active form. Much remains to be done before such changes can be interpreted.

The results presented here offer additional data. Tables 1 and 2 show that the total amount of RNA and proteins increased in the Deiters' nerve cells from the animals subjected to vestibular stimulation for 25 minutes/day for 7 days. The ratio RNA/proteins was maintained. This result confirms earlier results obtained with the UV-microspectrographic technique on the same type of material after vestibular stimulation (HAMBERGER and HYDÉN, 1949*a*, *b*). A series of UV-micrographs presented in these papers showed a 'diffuse' absorption of the nerve cell RNA from the stimulated animals. When such cells are stained, they exhibit typical chromatolysis, the most pronounced form of which is found in regenerating nerve cells. It may therefore be assumed that the change in the aggregation of the RNA which occurred during the considerable production of nucleoproteins shown in the present study, is typical for RNA which is engaged in rapid protein production.

As could be expected, concomitantly with the RNA increase after stimulation there occurred a significant increase of the proteins in the nerve cells. The cytochrome oxidase and the succinoxidase activities of the nerve cells also increased (Fig. 5). These data indicate that, not only an increase in the enzyme activities, but also an increase in the enzyme concentration occurred. In short, the protein production and the respiratory activity of the nerve cell have been increased as a result of the increased functional demands.

A possible role of the oligodendrocytes

As has been mentioned above, contradictory opinions can be found in the literature about the contribution of the neuroglia to the high oxidative metabolism of the CNS. When the two types of neutroglial cells are considered, earlier work clearly indicates that oligodendrocytes have a higher oxidative metabolism than have astrocytes (ABOOD *et al.*, 1952; POPE and HESS, 1957). POPE has concluded that the oxidative metabolism of oligodendrocytes may even approach that of the neurons. In an earlier study in this laboratory, the respiratory enzyme activity of the satellite cells (oligodendrocytes) was compared with that of the spinal ganglion cells (HYDÉN *et al.*, 1958). Determined per unit volume, the activity of the cytochrome oxidase was 0·9 for the nerve cells compared with 1·6 for the glial cells. The succinoxidase activity was 0·7 for the nerve cells and 5·1 for the satellite cells in the capsule. If the findings of LOWRY *et al.* (1957*a*) on the enzyme activity in the spinal ganglion cells and in their satellite cells are expressed on the same volume basis, the activities in the glial cells are much higher than those of the nerve cells.

* The resolving power at this wavelength was approximately 1000 Å.

The present data on Deiters' nerve cells and their 35–40 surrounding oligodendrocytes agree entirely with this conclusion (see Fig. 4). The oligodendrocytes may have a high oxidative metabolism of their own, or they may furnish the nerve cells with energy-rich compounds. If the latter, it would be appropriate to re-introduce the name 'trophocytes' for the oligodendrocytes.

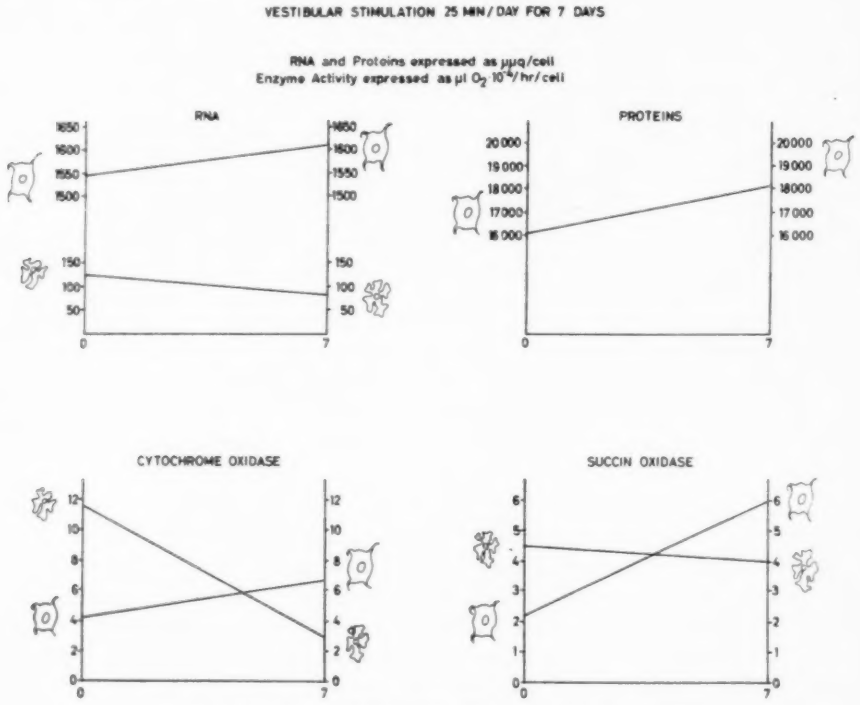

FIG. 5.—Survey of the results obtained in this study.

In the present experiments the amount of RNA and the respiratory enzyme activity decreased in the oligodendrocytes, but increased in the nerve cells after stimulation Provided that the blood supply is as favorable for the oligodendrocytes as for the nerve cells, this result indicates that the nerve cell and its oligodendrocytes are energetically linked in a functional system. If this view is confirmed, cytophysiological studies ought to take the nerve cell with its surrounding oligodendroglial cells as the functional unit of the central nervous system.

A tentative view of the function of the oligodendrocytes would be that, at an ordinary level of activity, the glia supply the nerve cell they serve with energy-rich compounds. Pinocytosis could be a likely transport mechanism. At higher functional demands, the nerve cell gets priority to the easily available energy for its protein production. The data presented here speak, however, against a direct transfer of enzymes. There is no direct relationship between the changes in the two types of cells. At intense function, the glial cells have therefore to resort to other sources of energy. An explanation of the high lipid content of the glia, may be that they can utilize lipoproteins and give priority to the nerve cells to utilize the glucose.

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SUMMARY

Fresh, isolated nerve cells, and the oligodendroglial cells surrounding them, have been analysed by suitable quantitative chemical and physical methods.

The mean RNA content of oligodendrocytes was 120 $\mu\mu$g compared with 1545 $\mu\mu$g in Deiters' nerve cells. The cytochrome oxidase and the succinoxidase activities were twice as high in the oligodendroglia as in an equal volume of nerve cells.

Vestibular stimulation for 25 minutes per day for 7 days produced a significant increase in the amount of RNA and a change of the absorption in the UV-micrograph. There was a concomitant increase in the amount of proteins per nerve cell and in the cytochrome oxidase and succinoxidase activities. It was concluded that stimulation caused an increase in the enzyme concentrations.

On the other hand, the amount of RNA and the respiratory enzyme activity decreased in the oligodendroglia on stimulation, showing that the nerve cell and the surrounding oligodendrocytes represent two interdependent functional systems which are linked energetically. The nerve cell plus the surrounding glial cells seem to constitute a functional unit in the central nervous system. As a working hypothesis it has been suggested that the nerve cells are given priority to the electron transport system when functional demands are increased.

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THE *IN VITRO* AND *IN VIVO* EFFECTS OF CHLORPROMAZINE ON BRAIN LIPID SYNTHESIS

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CHLORPROMAZINE has been reported to have a number of different actions *in vitro* and *in vivo* on brain lipid metabolism. MAGEE *et al.* (1956) demonstrated that the addition of chlorpromazine (CPZ), at a concentration of 10^{-4} M, to slices of guinea-pig brain produces an increased labelling of lipid P from inorganic ^{32}P. This effect was also observed when the final concentration of CPZ was as low as 10^{-5} M. Higher doses of CPZ induced a decrease in oxygen consumption (COURVOISIER *et al.*, 1953; PERUZZO and FORNI, 1953) followed by an inhibition of brain phospholipid biosynthesis.

The increase in phospholipid biosynthesis was observed only at the level of the 'inositol-P compounds', phosphatidic acid and phosphatidyl ethanolamine (ROSSITER, 1957). A similar effect was later observed (MAGEE, PRITCHARD and STRICKLAND. 1957) with guinea-pig brain slices when the radioactive precursors used were glycerol-1-^{14}C or serine-3-^{14}C but not with choline-1:2-^{14}C or acetate-1-^{14}C.

The results previously reported are not in agreement with the data obtained by CHRISTENSEN and WASE (1958, 1959) since under their experimental conditions, CPZ at a concentration of 10^{-4} M *in vitro*, inhibits the incorporation of fatty acids into phospholipids, preventing the fatty acid esterification with α-glycerophosphate, and inhibits ^{32}P incorporation into phospholipids in brain and liver homogenates, even at a concentration of 10^{-5} M.

In vivo experiments show that CPZ, at doses of 20–50 mg/kg, decreases ^{32}P incorporation and brain phospholipid turnover (WASE *et al.*, 1956; ANSELL and DOHMEN, 1956), and this effect is still apparent at dose levels down to 5 mg/kg (RICHTER, 1957). Lower doses were reported, on the other hand, to increase the incorporation of ^{32}P into phospholipids (CHRISTENSEN and WASE, 1959).

The following experiments were performed to investigate the effect of CPZ at different doses, both *in vitro* and *in vivo*, on cholesterol, glyceride and phospholipid fatty acid biosynthesis, from several ^{14}C-labelled precursors.

EXPERIMENTAL

Animals. Littermate Long-Evans male rats aged 10 days were used. The rats were sacrificed by decapitation, the brains and livers were rapidly excised and placed in ice-cold oxygenated Krebs–Ringer phosphate solution until used.

Materials. The labelled precursors used were: sodium acetate-1-^{14}C (specific activity 2 mc/mM), glucose-G-^{14}C (10 mc/mM) and mevalonic acid-2-^{14}C (0·6 mc/mM), obtained from the Radiochemical Centre; Amersham, U.K.

Chlorpromazine hydrochloride (2-chloro-10-(3-dimethylaminopropyl)-phenothiazine hydrochloride) was obtained from the Farmitalia Laboratories, Milano and used at the concentrations shown in the Tables.

Glucose was added at a concentration of 10 μ moles per ml, except when glucose-G-^{14}C was employed.

Preparation of tissues and incubation. In *in vitro* experiments 300 $\pm$ 3 mg of brain or liver slices of 400 μ thickness were incubated for 2 hr in Warburg flasks in 5 ml of Krebs–Ringer phosphate at pH 7·4 at 37·5° in 100% oxygen, as previously described (GROSSI, PAOLETTI and PAOLETTI, 1958).

In *in vivo* experiments, chlorpromazine was administered by the intraperitoneal route to the rats at doses of 3·5 mg/kg and 35 mg/kg, corresponding to *in vitro* concentrations of 10^{-5} M and 10^{-4} M respectively. Controls received the same volume of saline and all groups of animals were killed 1–4 and 24 hr after treatment, being fasted during this time. In all animals, brain and liver cholesterol and fatty acid biosynthesis was investigated.

Analysis. Substrate oxidation and incorporation into cholesterol and into phospholipid and glyceride fatty acids were measured. Substrate oxidation was determined by the method of LYON and GEYER (1954) counting the $^{14}CO_2$ collected in 20% KOH solution in the central wall of the Warburg flasks.

Lipid extraction and separation were carried out as follows: the lipids from brain and liver slices, at the end of the incubation time were extracted for 24 hr at room temperature using chloroform–methanol (2:1). The solvent was evaporated under vacuum and the residue was taken up using 15% chloroform in petroleum ether.

The acetone-soluble lipids were isolated by three extractions with acetone at room temperature, with the addition of $MgCl_2$. Both the acetone-soluble and the acetone-insoluble fractions (phospholipids) were taken up in N-KOH and saponified by heating for 4 hr.

The unsaponifiable fraction was separated by three extractions with ethyl ether and the fatty acids of both acetone-soluble and acetone-insoluble fractions, present as soaps in the aqueous phase, were liberated with 5 N-H_2SO_4 and separated by three extractions with low-boiling petroleum ether. The petroleum ether solution was then dehydrated with anhydrous Na_2SO_4; the purified fatty acids were plated according to POPJÁK (1950).

Cholesterol was isolated from the unsaponifiable portion of the acetone-soluble fraction as the digitonide and plated using a special centrifuge (PAOLETTI and PAOLETTI, 1957).

A Tracerlab gasflow counter was used for all ^{14}C determinations. The counting time was chosen to give a maximal error of 2%. Counter dead time, self-absorption and background radioactivity were allowed for in the usual way.

Results are reported as μmoles of the labelled precursors incorporated into one mg of cholesterol or fatty acids, and as μmoles of precursor oxidized per g of tissue.

RESULTS AND DISCUSSION

The rate of biosynthesis of cholesterol, acetone-soluble fraction and phospholipid fatty acids from acetate-1-^{14}C and substrate oxidation in the presence of different concentrations of chlorpromazine are shown in Table 1.

CPZ at a concentration of 10^{-3} M is a strong inhibitor of sodium acetate-1-^{14}C utilization in the brain of the rats. At concentrations of 10^{-4} and 10^{-5} M, on the other hand, the phospholipid fatty acid biosynthesis is increased about 2–2·5 times; this effect is only seen in growing rats.

The effect of CPZ on liver lipid biosynthesis under the same experimental conditions is also reported (Table 1b). Acetate utilization is markedly decreased by the addition of 10^{-3} M-CPZ, but the lower concentrations have no effect on phospholipid fatty acid biosynthesis.

The effect of CPZ on mevalonic acid 2-^{14}C metabolism in the brain (Table 2) seems to be very similar to its effect on acetic acid-1-^{14}C metabolism. The incorporation of mevalonic acid into brain phospholipid fatty acids in brain slices from young rats is significantly increased by CPZ, 10^{-4} and 10^{-5} M, while 10^{-3} M-CPZ has a depressing effect. Mevalonic acid oxidation is increased by the two lower doses of CPZ. Similar results are also obtained using glucose-G-^{14}C as a precursor of brain lipids (Table 3).

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In a second group of experiments (Tables 4 and 5) chlorpromazine was administered *in vivo* at doses of 3·5 and 35 mg/kg i.p. and, at different times after treatment, brain and liver slices were incubated *in vitro* and analysed for lipid biosynthesis.

TABLE 1.—THE *in vitro* EFFECT OF CHLORPROMAZINE ON ACETATE-1-^{14}C INCORPORATION INTO LIPIDS OF GROWING RATS

(a) Brain lipids

No. of animals	Treatment	$^{14}CO_2$*	Cholesterol†	Fatty acids†	
				Acetone-soluble fraction	Phospholipid
5	Controls	300·71 ± 17·10	13·15 ± 1·16	1·73 ± 0·38	0·42 ± 0·14
6	CPZ 10^{-3} M	29·69 ± 4·27‡	1·54 ± 0·26‡	0·38 ± 0·06‡	0·09 ± 0·01‡
6	CPZ 10^{-4} M	364·37 ± 15·67	14·70 ± 1·16	2·09 ± 0·26	0·90 ± 0·07‡
6	CPZ 10^{-5} M	390·45 ± 18·76§	14·03 ± 1·35	2·20 ± 0·33	1·03 ± 0·16‡
		(b) Liver lipids			
6	Controls	163·42 ± 18·52	2·54 ± 0·15	0·08 ± 0·005	0·02 ± 0·002
6	CPZ 10^{-3} M	98·33 ± 8·78‡	0·62 ± 0·05‡	0·03 ± 0·002‡	0·01 ± 0·001‡
6	CPZ 10^{-4} M	187·64 ± 16·41	2·85 ± 0·21	0·08 ± 0·005	0·02 ± 0·002
6	CPZ 10^{-5} M	157·05 ± 6·41	2·42 ± 0·11	0·09 ± 0·009	0·02 ± 0·001

Sodium acetate-1^{14}-C added at a concentration 1·06 μ moles/g tissue.
* 1×10^{-3} μ moles acetate oxidised per g tissue ± S.E.
† 1×10^{-3} μ moles acetate incorporated per mg of cholesterol or fatty acids ± S.E.
‡ $P < 0{\cdot}01$ in comparison with controls
§ $P < 0{\cdot}05$ in comparison with controls

TABLE 2.—THE *in vitro* EFFECT OF CHLORPROMAZINE ON MEVALONIC ACID-2-^{14}C INCORPORATION INTO BRAIN LIPIDS OF GROWING RATS

No. of animals	Treatment	$^{14}CO_2$*	Cholesterol†	Fatty acids†	
				Acetone-soluble fraction	Phospholipid
5	Controls	1·54 ± 0·35	3·06 ± 0·30	0·42 ± 0·04	0·05 ± 0·005
6	CPZ 10^{-3} M	1·08 ± 0·14‡	0·31 ± 0·07‡	0·07 ± 0·01‡	0·03 ± 0·007‡
6	CPZ 10^{-4} M	3·55 ± 0·48§	3·85 ± 0·37	0·44 ± 0·03	0·08 ± 0·005‖
5	CPZ 10^{-5} M	2·78 ± 0·50§	3·96 ± 0·19	0·42 ± 0·05	0·08 ± 0·005‖

Mevalonic acid-2-^{14}C added at a concentration of 3·03 μ moles/g of tissue.
* 1×10^{-3} μ moles mevalonic acid oxidised per g of tissue ± S.E.
† 1×10^{-3} μ moles mevalonic acid incorporated per mg of cholesterol or fatty acids ± S.E.
‡ $P < 0{\cdot}01$ in comparison with controls.
§ $P < 0{\cdot}02$ in comparison with controls.
‖ $P < 0{\cdot}05$ in comparison with controls.

CPZ at a dose of 3·5 mg/kg (corresponding to 10^{-5} M *in vitro*) increases brain phospholipid fatty acid biosynthesis from acetate-1-^{14}C (Table 4a) to about the same extent as has been observed *in vitro*. The maximum effect is obtainable between 1 and 4 hours after treatment and is not significantly increased after 24 hours. According to our *in vitro* data, CPZ has no effect on lipid biosynthesis in the liver (Table 4b).

TABLE 3.—THE *in vitro* EFFECT OF CHLORPROMAZINE ON GLUCOSE-G-^{14}C INCORPORATION INTO BRAIN LIPIDS OF GROWING RATS

No. of animals	Treatment	$^{14}CO_2$*	Cholesterol†	Fatty acids†	
				Acetone-soluble fraction	Phospholipid
6	Controls	389·51 ± 12·31	0·62 ± 0·04	0·02 ± 0·001	0·004 ± 0·0001
6	CPZ 10^{-3} M	53·68 ± 3·74‡	0·15 ± 0·01‡	0·007 ± 0·001‡	0·002 ± 0·0001‡
6	CPZ 10^{-4} M	395·95 ± 20·15	0·79 ± 0·05	0·02 ± 0·001	0·007 ± 0·0002§
6	CPZ 10^{-5} M	412·32 ± 21·78	0·50 ± 0·02	0·03 ± 0·001	0·007 ± 0·0003§

Glucose-G-^{14}C added at a concentration of 0·66 μ moles/g of tissue.
* 1×10^{-3} μ moles glucose oxidised per g of tissue ± S.E.
† 1×10^{-3} μ moles glucose incorporated into one mg of cholesterol or fatty acids ± S.E.
‡ $P < 0{\cdot}01$ in comparison with controls.
§ $P < 0{\cdot}05$ in comparison with controls.

TABLE 4.—THE *in vivo* EFFECT OF CHLORPROMAZINE ON ACETATE-1-^{14}C INCORPORATION INTO LIPIDS OF GROWING RATS

(a) Brain lipids

No. of animals	Treatment	$^{14}CO_2$*	Cholesterol†	Fatty acids†	
				Acetone-soluble fraction	Phospholipid
6	Controls	304·98 ± 22·09	10·92 ± 0·83	1·30 ± 0·07	0·35 ± 0·01
8	CPZ 3·5 mg/kg 1 hr	314·01 ± 33·25	11·47 ± 0·35	1·33 ± 0·04	0·74 ± 0·09‡
5	CPZ 35 mg/kg 1 hr	313·06 ± 36·18	12·58 ± 0·87	1·04 ± 0·04	0·22 ± 0·01
6	Controls	247·50 ± 23·51	6·29 ± 0·54	0·97 ± 0·11	0·21 ± 0·02
6	CPZ 3·5 mg/kg 4 hr	347·20 ± 27·79	8·12 ± 0·73	0·93 ± 0·05	0·46 ± 0·03‡
6	Controls	382·89 ± 48·40	4·84 ± 0·48	0·94 ± 0·09	0·35 ± 0·04
6	CPZ 3·5 mg/kg 24 hr	397·86 ± 39·19	4·29 ± 0·35	1·18 ± 0·09	0·42 ± 0·03

(b) Liver lipids

5	Controls	207·12 ± 22·56	4·96 ± 0·49	1·35 ± 0·09	0·19 ± 0·02
5	CPZ 3·5 mg/kg 1 hr	179·57 ± 15·20	3·61 ± 0·38	0·95 ± 0·09	0·14 ± 0·01
5	CPZ 35 mg/kg 1 hr	177·67 ± 18·76	4·20 ± 0·45	0·64 ± 0·07	0·08 ± 0·01

CPZ administered by intraperitoneal route 1–4 or 24 hr before death, both controls and treated animals being fasted during this time.
Sodium-acetate-1-^{14}C added *in vitro* at the concentration of 1·06 μ moles/g tissue.
* 1×10^{-3} μ moles acetate oxidised per g tissue ± S.E.
† 1×10^{-3} μ moles incorporated per mg of cholesterol or fatty acids ± S.E.
‡ $P < 0{\cdot}01$ in comparison with controls.

With mevalonic acid-2-^{14}C (Table 5), parallel effects with CPZ *in vitro* and *in vivo* have been obtained. In brain and liver of adult rats (90 days old) the effect of CPZ on cholesterol and fatty acid biosynthesis was investigated under similar experimental conditions. No significant increase due to CPZ was seen either *in vitro* or *in vivo*.

In previous work we have been able to identify a number of conditions where fatty acid synthesis in the brain are qualitatively or quantitatively different from that in other tissues.

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Mevalonic acid, the most important precursor of liver lipids (TAVORMINA *et al.*, 1956; GARATTINI, PAOLETTI and PAOLETTI, 1958), is poorly incorporated into brain fatty acids, both *in vivo* (GARATTINI, PAOLETTI and PAOLETTI, 1959*a*) and *in vitro* (GARATTINI, PAOLETTI and PAOLETTI, 1959*b*). In contrast, glucose is readily incorporated into the fatty acids of the young rat brain and much less readily utilized in

TABLE 5.—THE *in vivo* EFFECT OF CHLORPROMAZINE ON MEVALONIC ACID-2-^{14}C INCORPORATION INTO BRAIN LIPIDS

No. of animals	Treatment	^{14}CO*	Cholesterol†	Fatty acids†	
				Acetone-soluble fraction	Phospholipid
6	Controls	1·56 ± 0·40	1·75 ± 0·07	0·23 ± 0·02	0·11 ± 0·02
6	CPZ 3·5 mg/kg 1 hr	1·82 ± 0·28	1·83 ± 0·09	0·38 ± 0·09	0·17 ± 0·02‡

CPZ administered by intraperitoneal route 1 hour before death.
Mevalonic acid-2-^{14}C added *in vitro* at a concentration of 3·03 μ moles/g of tissue.
* 1×10^{-3} μ moles mevalonic acid oxidised per g of tissue ± S.E.
† 1×10^{-3} μ moles acid incorporated per mg of cholesterol or fatty acids ± S.E.
‡ $P < 0{\cdot}05$ in comparison with controls.

liver fatty acids (GROSSI, PAOLETTI and PAOLETTI, 1958). Brain lipids are synthesized from different precursors only in the growing animal: no appreciable synthesis from different precursors is demonstrable in the brain of adult rats (SRERE *et al.*, 1950; MCMILLAN *et al.*, 1957). Some experimental conditions, such as X-irradiation, considerably increase the biosynthesis of liver fatty acids, but have an opposite effect on brain fatty acids (GROSSI, PAOLETTI and PAOLETTI, 1959).

In experiments still in progress, it has been demonstrated that insulin, while increasing the rate of lipid synthesis from acetate and mevalonic acid in both brain and liver, has a much more pronounced effect on lipid synthesis in the brain.

The effect of chlorpromazine on brain phospholipid synthesis provides new evidence for the specific pattern of fatty acid synthesis in the brain.

SUMMARY

The effect of chlorpromazine on cholesterol, acetone soluble fraction and phospholipid fatty acid biosynthesis in rat brain and liver was investigated *in vitro* and *in vivo*.

Chlorpromazine, at a dose of 10^{-5} M, increased the incorporation of acetate-1-^{14}C mevalonic acid-2-^{14}C and glucose-G-^{14}C in brain phospholipid fatty acids. This effect was also evident after the *in vivo* administration of chlorpromazine (3·5 mg/kg i.p.).

Under the same experimental conditions chlorpromazine has no effect on liver lipid biosynthesis.

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THE DISTRIBUTION OF MONOAMINE OXIDASE AND SUCCINIC OXIDASE IN BRAIN

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MONOAMINE oxidase is a widely distributed enzyme which oxidatively deaminates a variety of aliphatic amines (BLASCHKO, 1952). It was first described in rat liver by HARE (1928) and was found to exist in the central nervous system by PUGH and QUASTEL (1937). It was indirectly implicated in central nervous system function by MANN and QUASTEL (1940) when they observed the inhibitory effect of amphetamine on this system. BIRKHAUSER (1941) studied the distributions of monoamine oxidase and cholinesterase in a few parts of the human brain. He found monoamine oxidase activity was highest in the thalamic and globus pallidus regions, slightly lower in the caudate nucleus and putamen, and lowest in the cortex. The variation in activity from the regions of highest to lowest activity extended only over a two-fold range. Cholinesterase activity was most concentrated in the candate nucleus and putamen, and considerably less concentrated in the thalamus and cortex. Here the variation was much greater, extending over a 35-fold range. In a more extensive study on dogs BOGDANSKI *et al.* (1957) found monoamine oxidase to be somewhat more active in the hypothalamic region, with little differences in activity in most other regions of the brain.

Monoamine oxidase is a particulate enzyme, believed to be localized in mitochondria (COTZIAS and DOLE, 1951). Succinic dehydrogenase and cytochrome oxidase are also well known mitochondrial enzymes, though their localization in brain is quite dissimilar from that of monoamine oxidase. These enzymes, according to BURGEN and CHIPMAN (1951) and to more recent histochemical studies by SHIMIZU and MORIKAWA (1957), predominate in the cellular areas of brain, especially the cerebral and cerebellar cortices. Because of the discrepant distribution of these mitochondrial enzymes it seemed worthwhile simultaneously to examine their distribution in different parts of the brain, both in whole homogenates and in mitochondrial preparations obtained by differential centrifugation.

METHODS

Studies were performed on dog, cow, and human brains. The dogs were anaesthetized with ether and killed by exsanguination, the skull was opened, and the brain removed intact. The brain was kept cold in ice, and sections were dissected in the cold room and homogenized in cold 0·3 M-sucrose. Cow brains were obtained fresh from the slaughterhouse, kept cool in an ice bucket, and handled in a similar fashion. Human brains were obtained at autopsy from individuals who had received no drugs acting on the central nervous system in the recent period prior to death and were devoid of any central nervous system pathology.

The dissection of the various portions of the brain was made on the basis of gross anatomical landmarks. The region called the corpus callosum also includes the septum pellucidum and fornix, and the hypothalamic region also contains the subthalamic area. These inclusions were necessary in order to obtain sufficient tissue for manometric analysis. All other dissections include only those regions listed in the Tables.

Succinic oxidase activity was determined on an aliquot of the fresh homogenate with the manometric procedure of SCHNEIDER and POTTER (1943). This was performed within a few hours of obtaining the brain in the studies of the dog and cow, but there was a considerably greater delay in the studies of human brain, usually amounting to more than a day.

About a third of each homogenate of cow brain tissue was dialysed for 36–48 hr against frequent changes of distilled water, and monoamine oxidase activity was determined in this fraction. The remainder of the homogenate was further diluted with 0·3 M-sucrose and subjected to differential centrifugation by the method of SCHNEIDER and HOGEBOOM (1950), with the modification that the mitochondrial fractions were obtained by centrifugation at 10,000 g for a 30-min period instead of the 10-min period recommended for rat liver. The mitochondrial fractions were assayed for succinic oxidase activity and the unused portions were dialysed in the same manner as the homogenates. Monoamine oxidase activity was determined on the dialysed fractions of both homogenates and mitochondria by the manometric technique with both tyramine and 5-hydroxytryptamine (5-HT) as substrates. The substrate was added from the sidearm after the flasks had been gassed with oxygen and thermoequilibrated, and a brief period of time for the evaluation of endogenous respiration had elapsed. The final concentration of the substrate in the flask was 0·02 M. Semicarbazide (0·044 M) was routinely employed to prevent further oxidation of the aldehyde, and, in some experiments, azide (0·009 M) was added to inhibit nonspecific endogenous respiration and the catalytic breakdown of the formed peroxide.

Initial studies on dog brain were performed in an analogous manner. However, the quantities of tissue available from several regions of a single brain were insufficient to yield accurate, reproducible values for monoamine oxidase activity in both whole homogenate and mitochondrial preparations. In subsequent studies, all the brain tissue was used to evaluate enzymic activity of the whole homogenate, and only the data from these latter experiments are presented here.

In human brain, a broader analysis of the substrate specificity of monoamine oxidase was performed on mitochondrial preparations obtained from each region. Substrates employed were: tyramine, 5-HT, tryptamine, dopamine, L-epinephrine, L-norepinephrine, phenylethylamine, DL-β-phenylethanolamine, *n*-amylamine, and *iso*amylamine. Mitochondrial protein was determined by the method of LOWRY *et al.* (1951).

RESULTS

Activity of succinic oxidase and monoamine oxidase in dog brain homogenate. Succinic oxidase activity was highest in the cerebral and cerebellar cortices and lowest in the white matter of the cerebellum and subcortex. Intermediate activities were observed in the thalamus, hypothalamus, corpus callosum region, and brain stem. Monoamine oxidase activity, in contrast, was highest in the hypothalamic and thalamic regions, with little difference in enzyme activity in the other regions of the brain. In all regions of the brain, tyramine was oxidized at a rate 1·9–2·5 times that of 5-HT (mean = 2·1 $\pm$ 0·2). The quantity of white matter from the cerebellum was insufficient to permit accurate monoamine oxidase studies by the method employed, and this information is omitted from Table 1.

Activity of succinic oxidase and monoamine oxidase in bovine brain homogenate. The distribution of succinic oxidase activity in cow brain homogenate was similar to that in dog brain, being highest in the cerebellar and cerebral cortices, and lowest in the subcortex, cerebellar white matter, and spinal cord. Intermediate activity was observed in the other regions of the brain. Monoamine oxidase was most active in the thalamus and hypothalamus. It occurred in lowest amounts in both regions of the cerebellum, the subcortex, and spinal cord. The ratio of the rates of enzymic oxidation of tyramine and 5-HT varied from 2·0–3·3 for the various regions of the brain (mean = 2·6 $\pm$ 0·5) (Table 2).

Activity of succinic oxidase and monoamine oxidase in bovine brain mitochondria. The distribution of the activity of succinic oxidase in the mitochondrial fractions

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was similar to that in the original homogenates. In general, activity was somewhat lower in the mitochondrial fraction, owing in part to the incomplete recovery of the mitochondria in the fractionation procedure, and in part to some deterioration of the enzyme during the fractionation steps. Similarly, the relative distribution of monoamine oxidase in the mitochondrial fractions was not appreciably different from that in the original homogenates. The recovery of monoamine oxidase activity in

TABLE 1.—SUCCINIC OXIDASE AND MONOAMINE OXIDASE ACTIVITIES OF DOG BRAIN HOMOGENATE

Brain section	Substrate oxidized*		
	Succinate	Tyramine	5-HT
Cortex	5018	102	53
Subcortex	2195	91	37
Corpus callosum	2912	112	53
Thalamus	3763	156	81
Hypothalamus	3225	170	74
Brain stem	2912	115	60
Cerebellum-grey	4300	106	48
Cerebellum-white	2307	—	—

Each figure represents the mean of three experiments.
* μl O_2 consumed/g tissue per hr.

TABLE 2.—SUCCINIC OXIDASE AND MONOAMINE OXIDASE ACTIVITIES OF BOVINE BRAIN HOMOGENATE

Brain section	Substrate oxidized*		
	Succinate	Tyramine	5-HT
Cortex	3069	136	57
Subcortex	717	82	28
Corpus callosum	2106	144	44
Thalamus	2520	213	69
Hypothalamus	2710	198	66
Brain stem	2307	122	53
Spinal cord	1635	99	41
Cerebellum—grey	4323	69	35
Cerebellum—white	1120	52	24

Each figure represents the mean of 3 experiments.
* μl O_2 consumed/g tissue per hr.

the various fractions ranged from 65–104 per cent of the activity in the respective homogenates, based on the ability to oxidize tyramine. The ratio of the rates of oxidation of tyramine and 5-HT varied from 1·7–3·4 for the various brain regions (mean = 2·6 $\pm$ 0·5) (Table 3).

Relative activities of succinic oxidase and monoamine oxidase in the various brain fractions. For dog brain, the ratio of activities of 'tyramine oxidase' to succinic oxidase ranged from 0·020 for cerebral cortex to 0·053 for hypothalamus. The ratio of activities of '5-HT oxidase' to succinic oxidase ranged from 0·011 for cerebral

TABLE 3.—SUCCINIC OXIDASE AND MONOAMINE OXIDASE ACTIVITIES OF BOVINE BRAIN MITOCHONDRIA

Brain section	Substrate oxidized*		
	Succinate	Tyramine	5-HT
Cortex	2598	96	32
Subcortex	538	57	26
Corpus callosum	1568	102	30
Thalamus	1885	142	53
Hypothalamus	1635	131	57
Brain stem	1568	106	47
Spinal cord	986	103	50
Cerebellum—grey	2867	45	20
Cerebellum—white	1053	48	29

Each figure represents the mean of 3 experiments.
* μl O_2 consumed/g tissue per hr.

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TABLE 4.—RELATIVE ACTIVITIES OF SUCCINOXIDASE AND MONOAMINE OXIDASE IN SECTIONS OF DOG BRAIN

Brain section	'Tyramine oxidase' / Succinoxidase	'5-HT oxidase' / Succinoxidase
Cortex	0·020	0·011
Subcortex	0·041	0·017
Corpus callosum	0·038	0·018
Thalamus	0·041	0·022
Hypothalamus	0·053	0·023
Brain stem	0·039	0·021
Cerebellum—grey	0·025	0·011

TABLE 5.—RELATIVE ACTIVITIES OF SUCCINOXIDASE AND MONOAMINE OXIDASE IN SECTIONS OF BOVINE BRAIN

Brain section	'Tyramine oxidase' / Succinoxidase		'5-HT oxidase' / Succinoxidase	
	Homogenate	Mitochondria	Homogenate	Mitochondria
Cortex	0·044	0·037	0·019	0·012
Subcortex	0·114	0·106	0·039	0·048
Corpus callosum	0·068	0·065	0·021	0·019
Thalamus	0·085	0·075	0·027	0·028
Hypothalamus	0·073	0·080	0·024	0·035
Brain stem	0·053	0·068	0·023	0·030
Spinal cord	0·061	0·104	0·025	0·051
Cerebellum—grey	0·016	0·016	0·008	0·007
Cerebellum—white	0·046	0·046	0·021	0·028

cortex to 0·023 for hypothalamus (Table 4). In bovine brain, the variation of ratios was more striking; for 'tyramine oxidase' to succinic oxidase, the ratios ranged from 0·016 for the cerebellar cortex to 0·114 for the subcortex, and for '5-HT oxidase' to succinic oxidase, from 0·008 for cerebellar cortex to 0·039 for subcortex. The ratios of the two enzymes were not appreciably different when the homogenate and mitochondrial preparations from the same regions were compared. Where a difference was observed, it was generally due to a greater fall in succinic oxidase activity in the mitochondrial preparation, presumably due to a deterioration of this more labile enzyme system. This seemed to be especially true for the bovine spinal cord, where the succinic oxidase activity in the mitochondrial preparation had fallen to almost half of that in the homogenate without any appreciable change in the monoamine oxidase activity (Table 5).

TABLE 6.—RELATIVE RATES OF OXIDATION OF SEVERAL AMINES BY MITOCHONDRIAL PREPARATIONS FROM HUMAN BRAIN*

Substrate	Rate of oxidation†
n-Amylamine	11 ± 2
*Iso*amylamine	153 ± 40
Phenylethylamine	4 ± 2
Phenylethanolamine	22 ± 5
Dopamine	83 ± 22
Norepinephrine	30 ± 12
Epinephrine	22 ± 14
Tryptamine	14 ± 5
5-Hydroxytryptamine	20 ± 7

* Based on tyramine oxidation = 100.
† Mean ± S.D.

Monoamine oxidase activity in different regions of human brain: Succinic oxidase activity in preparations from human brain was very low. This was believed to be due to the postmortal deterioration of this enzyme, and consequently studies on this enzyme were not further pursued.

Because of the large amount of material available, it was possible to perform a broad substrate analysis on different regions of the human brain, in mitochondrial fractions obtained by differential centrifugation. In the several regions of the brain examined, the substrate specificity pattern was similar. *Iso*amylamine, tyramine, and dopamine were most readily oxidized. Phenylethylamine was poorly oxidized, and β-phenylethanolamine was only a slightly better substrate. *n*-Amylamine and tryptamine were poor substrates, and 5-HT was only slightly better oxidized. Epinephrine and norepinephrine were oxidized at intermediate rates. The similar patterns of enzymic activity observed for the various regions of the brain justified the grouping together of the data, and the mean rate of oxidation of each substrate relative to tyramine is presented in Table 6.

Mitochondrial preparations from the thalamus, hypothalamus, and putamen and globus pallidus were most active on the basis of the weight either of original tissue or of mitochondrial protein. The cerebellum contained the lowest monoamine oxidase activity (Table7).

Distribution of monoamine oxidase and succinic oxidase in various fractions obtained by differential centrifugation. Neither the microsomal fraction nor the final supernatant liquid contained appreciable amounts of either enzyme in any of the tissues examined. The enzymes were found exclusively in the mitochondrial fractions and in the cell debris. The activity in the cell debris presumably is present in mitochondria of cells not disrupted during the process of homogenization.

TABLE 7.—MONOAMINE OXIDASE ACTIVITY OF REGIONS OF HUMAN BRAIN*

Brain section	Activity†
Cortex	9·8
Subcortex	9·1
Corpus callosum	9·6
Thalamus	19·0
Hypothalamus	16·6
Putamen and globus pallidus	16·3
Cerebellum	6·8
Brain stem	10·3

The values presented are the means of 2 experiments.
* Substrate-tyramine.
† μl O_2 consumed/mg mitochondrial protein per hr.

DISCUSSION

The relative distribution of succinic oxidase of both dog and cow brain was quite similar in the several regions of the brain, being highest in the more cellular grey matter and lowest in the relatively acellular white matter. Monoamine oxidase activity, on the other hand, was most highly concentrated in the thalamic and hypothalamic regions for dog, cow, and human brain. Both enzymes were found to be chiefly localized in the mitochondrial fractions of the various regions of the brain, with a smaller proportion in the cell debris fraction. In general, the activity of the two enzymes appeared to become concentrated in the mitochondrial fractions in similar proportions.

The substrate specificity patterns of monoamine oxidase of mitochondrial preparations obtained from the several regions of human brain all appear to be similar. Moreover, the pattern is essentially the same as that observed for homogenates of whole human brain (WEINER, 1959).

It thus seems quite clear that these two enzyme systems are located in particles with similar centrifugation characteristics, yet are present in different proportions in some parts of the brain. There are several possible explanations for these observations. It has been suggested that there is more than one monoamine oxidase in brain tissue and that these 'enzymes' have dissimilar distributions (BOGDANSKI *et al.*, 1957). The similarity of the substrate patterns of monoamine oxidase in different regions of the brain, plus the localization of most or all of the activity in particles, argue against this hypothesis.

Another postulation is that there are several particles which have the sedimentation characteristics of mitochondria, but which are biochemically quite different. Thus, monoamine oxidase could exist in one type of particle and succinic oxidase could be

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present in the more classical mitochondrial particle. Varying proportions of these particles in different portions of the brain could account for the results obtained. NOVIKOFF *et al.* (1953) and KUFF and SCHNEIDER (1954) have demonstrated by differential centrifugation that particles from liver homogenates with the sedimentation characteristics of mitochondria are biochemically heterogeneous. In brain mitochondrial preparations, BRODY and BAIN (1952) have observed a morphological heterogeneity of the particles even greater than that in liver.

A third possible explanation for the present results, which is really a modification of the second, is that the mitochondria themselves are heterogeneous particles. PALADE (1952) and others have shown by electron microscopy that particles believed to be mitochondria have different morphological characteristics in different tissues. The major differences appear to be in the size, shape, and the ratio of intramitochondrial ridges, or cristae, to matrix. It has been postulated that these cristae house the enzymes of oxidative metabolism including the succinic oxidase system. In a few tissues examined, there appears to be a correlation between succinic oxidase activity and the number of these cristae per mitochondrion. KUFF and SCHNEIDER, in their studies of the heterogeneity of liver mitochondria, were unable to isolate particles which completely lacked any of the enzymes of the whole fraction; only the relative amounts of each enzyme varied in the subfractions. This suggests that the particles may differ in a quantitative way rather than a qualitative one.

The data presented here suggest that the mitochondrial fraction of brain is also biochemically heterogeneous, and that the biochemical differences are quite marked when mitochondria from different regions of the brain are compared. More extensive biochemical and morphological information about these particles in various regions of the brain is required before one can say whether these two enzymes reside in the same particles, which differ in different areas of the brain, or whether they occur in morphologically distinct particles which possess similar sedimentation characteristics.

SUMMARY

The distribution of two particulate enzymes, monoamine oxidase and succinic oxidase, has been studied in various regions of dog, cow, and human brain. Succinic oxidase is most active in the cellular areas of the brain, especially the cerebellar and cerebral cortices. Monoamine oxidase is most active in the thalamic and hypothalamic regions. Both enzymes are found predominantly, and in similar proportions, in the mitochondrial fraction. Neither is detectable in the microsomal fraction or in the cell supernatant fluid. The behavior of monoamine oxidase toward different substrates in the various regions of the brain is similar. These results support the hypothesis that the mitochondrial fractions from various regions of the brain are biochemically heterogeneous. Either these two enzymes reside in distinct particles or the analogous particles contain different amounts of each enzyme in different regions of the brain.

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Papers to be published in future issues

J. H. WELSH and M. MOORHEAD: The quantitative distribution of 5-hydroxytryptamine in the invertebrates, especially in their nervous systems.

M. NIEMI and E. MERENMIES: Cytochemical localization of the oxidative enzyme systems in the retina—I. Diaphorases and dehydrogenases.

M. NIEMI and E. MERENMIES: Cytochemical localization of the oxidative enzyme systems in the retina—II. Cytochrome oxidase.

R. L. FRIEDE: Histochemical investigations of succinic dehydrogenase in the central nervous systems—V. The diencephalon and basal telencephalic centres of the guinea pig.

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K. F. GEY and A. PLETSCHER: Activity of monoamine oxidase in relation to the 5-hydroxytryptamine and norepinephine content of the rat brain.

L. D. MACLEOD: The distribution of the arylamino groups in the brain of the albino rat following administration of *p*-aminobenzoic acid.

E. L. BENNETT, M. R. ROSENWEIG, D. KRECH, A. OHLANDER and H. MORIMOTO: Cholinesterase activity and protein content of rat brain.

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HISTOLOGICAL DEMONSTRATION OF HYALURONIC ACID IN THE CENTRAL NERVOUS SYSTEM*†

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MUCOPOLYSACCHARIDES (MP)‡ have been known to exist in tissue for several decades, but it is only within the past ten years that interest in MP has extended to the nervous system (ABOOD and ABUL-HAJ, 1956; BRANTE, 1957). The presence of MP in the central nervous system has been demonstrated chemically by BRANTE (1957, 1958), but an extensive characterization has not been accomplished. The preponderance of evidence for MP in the central nervous system, however, is histologic.

One of the earliest studies of MP in the CNS is that of FREIBERG and RINGERTS (1956), who demonstrated that upon the injection of ^{35}S into rat embryos, the uptake of sulphur was very low in both peripheral nerve and CNS up to 10 days, but at 17 to 18 days there was a fairly high uptake in the *corpus striatum* and certain cortical layers, as judged by PAS-staining properties. Neuroglia, particularly astrocytes, were found to contain PAS-positive granules which were not cerebroside or lecithin (LUMSDEN, 1958). WOLMAN (1956, 1957) reported that during degeneration of the myelin sheath, MP is released, presumably from the myelin. HESS (1955) suggested that MP may be related to the blood–brain barrier, since the disappearance and reappearance of the barrier correlated well with the reappearance of the PAS-staining reaction. Insofar as H-ase did not alter PAS staining, the material was probably not HA. OZELLO *et al.* (1958), on the other hand, did not detect PAS staining comparable to the ground substance of other tissues, except in the walls of blood vessels.

The use of H-ase in demonstrating the presence of MP in the central nervous system has been surprisingly limited. H-ase produced a greater diffusion of trypan blue through either normal or traumatized brain tissue (ARTETA, 1956), and facilitated the penetration of Indian ink around the nerve cells (BAIRATI, 1953). FREEDMAN (1953) also was able to demonstrate a difference in the metachromatic staining of brain sections with thionine after treatment with H-ase, particularly in the 'intercellular matrix of the grey matter.'

Since a previous study had demonstrated the presence of HA in the axoplasm and neurilemma sheath of amphibian and mammalian nerve, there was reason to believe that the neurons of the central nervous system should likewise contain the material. The present study is devoted to an extensive histological exploration of MP in the mammalian brain and definitely establishes the existence of an MP within the neurons, which is readily attacked by testicular H-ase. Furthermore, it will be

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† This research was part of a dissertation for the partial fulfilment of the Ph.D. degree by the senior author in the Department of Physiology, University of Illinois College of Medicine.

‡ The following abbreviations are used: MP, mucopolysaccharides; HA, hyaluronic acid; H-ase, hyaluronidase; PAS, periodic acid Schiff stain; TRU, turbidity reducing units.

shown that a considerable amount of the PAS-positive material within the neurons, as well as within the neurophil, is not HA.

METHODS

Histological procedures. Prior to removal, the brains were perfused by way of the carotid arteries with 3 l. of acacia solution, followed by 3 l. of 10% formalin, after the method of KOENIG *et al.* (1945). The embryonic brains were perfused by means of a 15 or 20 gauge needle placed in the left ventricle and incision of the right atrium. The tissues were then allowed to fix for at least 48–72 hr in 10% formalin.

A double embedding technique, described by LA VELLE and LA VELLE (1958), was used for the histological preparations. The tissues were cut to approximately 1 cm^2 and kept for 60 min with gentle agitation in each of the following ethanol concentrations successively: 30%, 50%, 70%, 95%, and 100%. After exposure to ethanol–ether (1 : 1) for 60 min, the tissues were kept successively in 2%, 4%, and 6% colloidin for a period of 2 to 3 days at each concentration. After hardening in 6% colloidin in chloroform, the blocks of tissue were cut, left overnight in benzene to remove the chloroform and other solvents, and placed in melted Fisher tissue mat for 24 hr at 60°. The sections were cut to a thickness of 10 μ. Before treatment with H-ase and other enzymes, the slides were kept in an oven at 55°–60° overnight to fix the sections securely to the slide. To remove the paraffin, the slides were washed for 3 min successively in xylene, xylene–ethanol (1 : 1). and ethanol, To ensure complete removal of lipids, the slides were allowed to remain for 20 min in ethanol–ether (1 : 1), and then dehydrated in ethanol, as described above. Alternate sections were incubated for 20 hr in a medium containing testicular H-ase* (150 TRU/ml), 0·1 M-NaCl, and 0·05 M-sodium acetate at pH 6. The control medium contained alternate sections adjacent to the experimental, and bovine albumin was substituted for H-ase. Ribonuclease was used at a concentration of 1 : 50,000, which was twice that recommended by LILLIE (1947) as necessary for the removal of ribonucleic acid. The slides were incubated in the enzyme for 90 min at 37°.

The technique of RINEHART and ABUL-HAJ (1951) was used for staining with minor modifications. Several variations of the staining procedure were made to determine the effect upon the stainability of the cells. The first modification was to eliminate ferric ammonium glycerate, and to replace it with a bath containing 4 N-acetic acid. The procedure was then carried out as described by RINEHART and ABUL-HAJ (1951). Another change was to substitute 0·5% HCl for potassium ferrocyanide. Next, an aqueous Schiff reagent was substituted for the alcoholic Schiff reagent. The last modification was to immerse the slides in concentrated H_2SO_4 for 60 sec and then to rinse them thoroughly in distilled water to remove the excess acid. The tissues were then placed in 3% acetic acid and stained in the usual manner. In each one of the modifications, a control group of slides was stained in the usual manner. Photographs were made with a Kodachrome professional Type A film, without filters.

Effect of prolonged electrical stimulation on MP. Two bullfrog sciatic nerves were placed in Ringer solution. One was the control, and the other was stimulated at a frequency of 100 cycles/sec for 16 hr with continuous recording of the action potential. Both were placed in formalin for 24 hr and prepared as before for sectioning. Lobster giant axons were isolated, as described by TOBIAS† *et al.* (1955), immersed in 1000 TRU of H-ase, and stimulated for 8 hr with continuous recording. Control fibres were run simultaneously. The nerves were fixed in formalin, sectioned and stained.

Effect of intracisternal administration of H-ase. A group of 10 adult rabbits were given a series of 5 intracisternal injections of 5 mg of H-ase (8500 TRU) at equal intervals over a period of 4 days. A total of 5 control animals received 5 mg of bovine albumin instead of the enzyme. The intracisternal tap was performed under light ether anaesthesia. The behaviour of the rabbits was observed carefully during this period, in an effort to detect significant changes in general behaviour. Changes in reflexes, muscle tonus, respiration, heart rate, and response to various stimuli were among some of the determinations made. The rabbits were sacrificed and their brains perfused as described previously and prepared for sectioning.

* The testicular H-ase had an activity of 1700 TRU/mg. This material was assayed and found to be free of proteolytic enzymes, amylase, and RNA-ase. On some histological sections a testicular H-ase with 20,000 TRU/mg was used with essentially similar results. This latter material was obtained from Dr. ALBERT DORFMAN.

† The authors are indebted to Dr. JULIAN TOBIAS for his assistance in the preparation of lobster giant axons.

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Regeneration of damaged nerves. A group of 15 albino rabbits were anaesthetized with ether, an incision was made over the hamstrings, and the sciatic nerve isolated and cut 5 cm beyond the imaginary line drawn between the greater trechanter and the ischeal tuberosity. A small portion (1 cm) of nerve was removed to prevent bridging by the regenerating fibres. Skin clips were applied, and 200 mg of penicillin G were given intramuscularly. One animal was sacrificed immediately as a control, and animals were killed after intervals of 1–84 days. After perfusion, the sciatic nerves were removed along with the spinal-cord section containing their dorsal and ventral roots. They were placed in formalin for at least 24 hr and embedded and prepared for sectioning and staining. One slide of the cord material was stained with thionine and one with the usual iron and PAS stain, whereas the nerves were stained with iron and PAS only.

Effect of H-ase on dorsal root ganglion. The dorsal root ganglion isolated from the bullfrog was immersed in H-ase (1500 TRU/ml) and the resting potential and spike potential were measured periodically for a period of 8–10 hr.

RESULTS

Normal pattern of staining. Upon gross examination, the central nervous system of the embryos appeared blue throughout, the blue staining appearing as early as the tenth day of gestation. In adult brains of all species examined, the MP was distinctly apparent, grossly. Dog and rabbit brains were examined in all of the basic anatomical areas: spinal cord, upper and lower medulla, pons, inferior and superior colliculi and midbrain tegmentum, hypothalamus, massa intermedia of the thalamus, caudate nucleus, motor and pyriform cortex. All areas stained grossly with much the same pattern, the grey matter absorbing more of the iron than the white matter, with the latter staining more intensely with PAS.

Microscopically, the brains of all of the animals had the same basic cytologic pattern. The glial nuclei were only rarely visible, if at all, and no processes or glial podia were stained with Prussian blue. The neurons, on the other hand, stained well with the iron stain, with the nucleus staining a darker blue than the cytoplasm. The staining of the cytoplasm was more intense at the periphery of some cells, and in many cells blue granulations were apparent throughout the cytoplasm. The periphery of the cells would stain with PAS, whereas the cytoplasm would only occasionally show a faint PAS-staining, largely as granulations. In the pyriform cortex, the blue colour in the soma was continuous with the axon. The medullary sheaths and neuropil stained diffusely with PAS. In the embryonic nervous system, the blue stain was first concentrated in the periphery of the cell, but became more diffuse intracellularly with age.

Histological illustrations. The neuronal cell bodies of the brains of 19-day-old rat embryos were stained an intense blue, particularly within the cytoplasm (Fig. 1a); occasionally, a blue-stained neuroblast was distinctly evident. Upon treatment with H-ase, the cytoplasmic MP almost completely disappeared, whereas the nucleus appeared unchanged (Fig. 1b). The presence of MP was especially evident throughout the perikaryon and neuronal processes of the large pyramidal cells of the upper medulla (Fig. 2a). After H-ase digestion, the more diffuse cytoplasmic MP almost vanished, whereas the nuclear material again was only slightly affected. Although much of the granulated MP in the cytoplasm was removed by the enzyme, an appreciable amount still remained, particularly in the perinuclear region. The differentiation between the red PAS-staining material and the intracellular MP was evident after H-ase digestion. The intensification of the PAS staining is probably attributable to the fact that the Prussian blue stain either normally interfered with PAS or concealed the PAS.

Intracisternal administration of H-ase resulted in the removal of MP from the cytoplasm of lower motor neurons, as well as from the surrounding neuropil (Figs. 3a, 3b). Again, the nuclear material was unaffected, whereas the neuronal processes were hardly visible after removal of the blue-staining material.

Disappearance of the blue-staining material was evident during degeneration of the lower motor neurons of adult rabbit brain 10 days following nerve section (Figs. 4a, b). The blue material in the peripheral region of the neuron, as well as in the processes, vanished more readily during degeneration (Figs. 4a, b). It can readily be seen that the Nissl substance was especially rich in MP and that the MP vanished with progressive degeneration and chromatolysis. Noteworthy was the disappearance of the blue stainability of the nucleolus and nuclear membrane.

A lower magnification of the dog cerebellum revealed the differential staining in the various layers and various types of cell (Figs. 5a, b). The large Purkinje cells, along with the apical dendrites, stained most intensely for MP. Again, the nucleolar blue-staining material was only slightly affected by H-ase. The granular cells of the molecular layer of cerebellum stained positively for MP, although weakly in comparison with the Purkinje cells. The staining was largely confined to the nuclei of the granular cells, which were largely devoid of cytoplasm. The appearance of the red PAS was particularly evident in the cerebellum after treatment with H-ase. At lower magnification, the intense blue staining of the anterior horn cells of the dog's spinal cord was in effective contrast to the surrounding neuropil. Again, the dendritic processes were deeply stained (Figs. 6a, b), whereas the axons were barely visible. Neither the neuroglia nor myelin appeared to stain blue. Of particular significance was the fact that the nucleolar and nuclear blue disappeared almost completely after the enzyme, in contrast to the situation with regard to the previous material.

The MP in the dog's medulla was confined largely to the neuronal cell bodies and their dendrites, whereas again the axons showed only traces of the material (Fig. 7). At lower magnification, the hippocampus was seen to be diffusely stained with the most intense staining in the larger neurons (Fig. 8). Upon treatment with H-ase, the blue-staining material disappeared almost completely from all regions (Figure not shown). The capillary walls appeared to be devoid of any MP.

Modification of staining, resulting from enzyme digestion. As a result of H-ase digestion *in vitro*, there was almost complete removal of the blue coloration from the cytoplasm of the neuron, whereas the nucleus was largely unchanged. With the disappearance of the blue stain after H-ase digestion, the PAS staining was considerably intensified. The brains of rabbits chronically treated with H-ase showed quite obvious changes throughout, although no behavioural changes were observable. Throughout the CNS there was a loss of MP from the cytoplasm of the neurons, though the changes were not quite as obvious as those *in vitro*. Again, the cytoplasm and axon stained positively with Prussain blue, whereas the nucleus did not. There was a disappearance of MP in the lobster giant axons immersed in H-ase, yet the axon continued to conduct nerve impulses as well as did the control.

Modification of staining resulting from prolonged stimulation. Upon continued stimulation, there were no detectable difference in staining properties between control and experimental isolated bullfrog nerves. Nor was there a noticeable difference in the brains of mice treated with the various convulsant agents such as metrazol and methyl guanidine.

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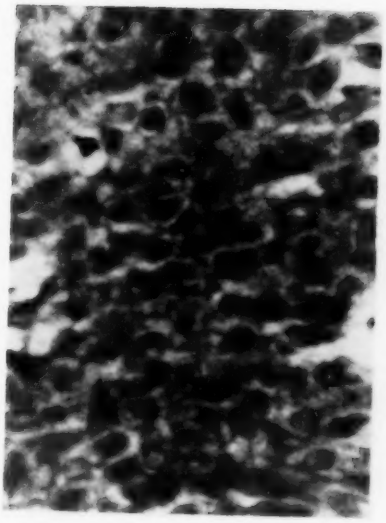

FIG. 1a.—Brainstem, 20 day rat embryo

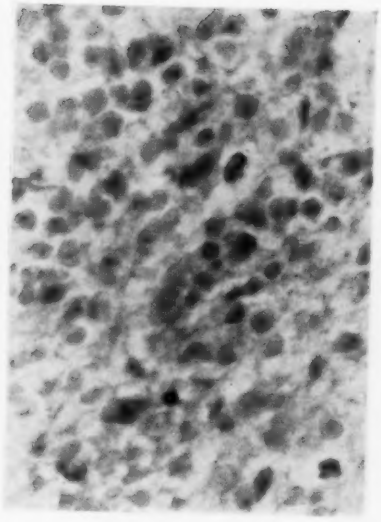

FIG. 1b.—Same as 1a, except digested with H-ase

FIG. 2a.—Large pyramidal cell, upper medulla of adult dog

FIG. 2b.—Same as 2a, except H-ase digested

Magnification: 280×

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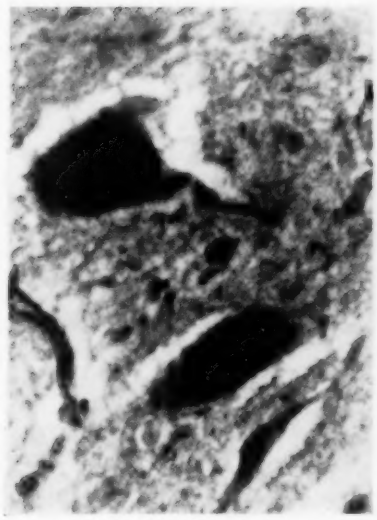

FIG. 3a.—Lower motor neurons, cervical cord of adult dog

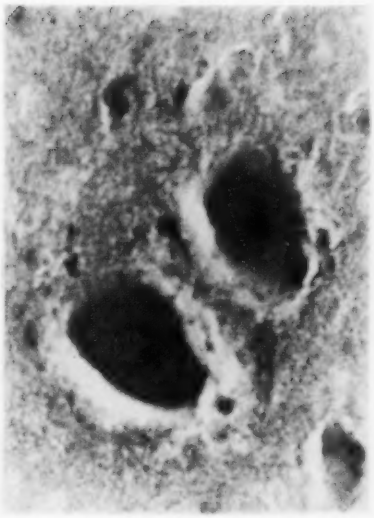

FIG. 3b.—Same as 3a, except H-ase digested

FIG. 4a.—Lower motor neuron, adult rabbit

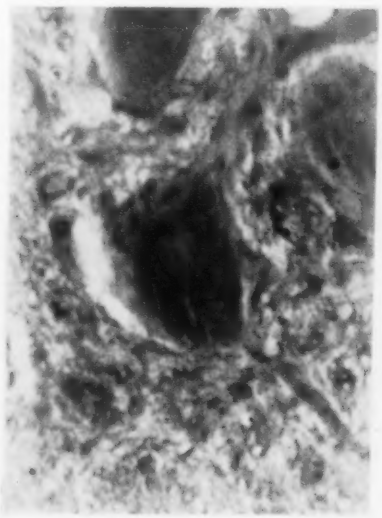

FIG. 4b.—Same as 4a, 10 days after nerve section.

Magnification: 280×

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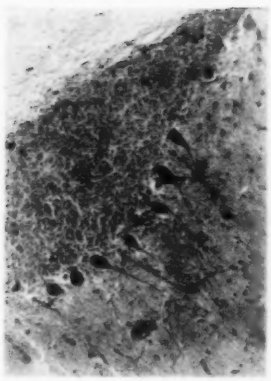

FIG. 5a.—Cerebellum, dog.

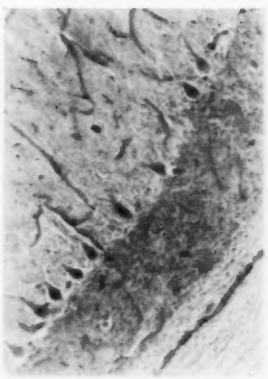

FIG. 5b.—Same as 5a, except H-ase digested.

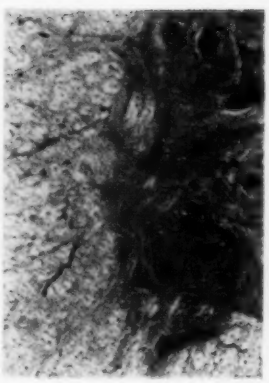

FIG. 6a.—Anterior horn cells, spinal cord of dog.

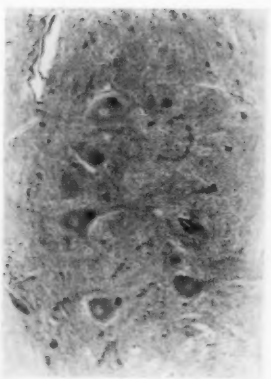

FIG. 6b.—Same as 6a, except H-ase digested.

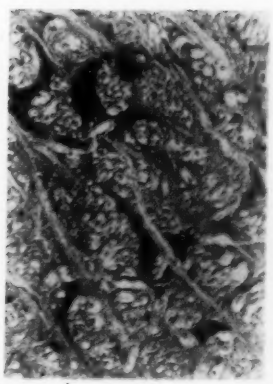

FIG. 7.—Medulla, dog.

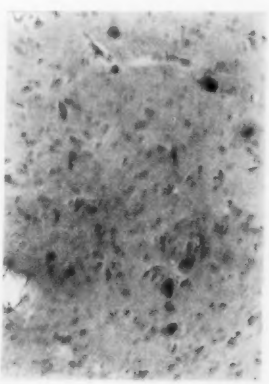

FIG. 8.—Hippocampus, dog.

Magnification: 50×

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Modification of staining resulting from sciatic nerve section. During nerve regeneration, normal cells which were stained with thionine or with Prussian blue exhibited a granulated appearance resembling Nissl substance. Abnormal cells, on the other hand, which were undergoing chromatolysis (as revealed by thionine), showed a disappearance not only of the granular Nissl pattern, but also of the blue stain in general. With the return of the normal Nissl granulation with thionine staining, there was also a return of the normal granules with the RINEHART–ABUL-HAJ stain. In peripheral nerve, the appearance of blue staining at the regenerating tip of the axoplasm indicated that MP appears quite early in the formation of new axoplasm.

Modification of staining resulting from changes in staining technique. As a result of RNA-ase digestion, there was no loss of the Prussain blue stain. Acetate buffer alone, or albumin in acetate buffer, in no way affected blue staining. Sulphation with concentrated H_2SO_4 resulted in a complete loss of PAS staining and a marked increase in the intensity of the coloration from the Prussian blue stain. After sulphation the neurons were still more intensely stained than the surrounding neuropil. When an alcoholic solution of Schiff stain was used, the usual blue and fuschia coloration was obtained; however, when an aqueous solution was used, the normal Schiff coloration was absent. In the absence of either ferric ammonium glycerate or potassium ferrocyanide, the histological sections appeared as if they had been treated with H-ase.

Physiologic and pharmacologic results. The results of the physiological and pharmacological studies have been largely negative. Immersion of the frog dorsal root ganglion into H-ase for as long as 10 hr resulted in no change in either the resting or action potential. The lobster giant axon, as well as the dorsal root ganglion preparation, exhibited virtually normal electrical properties, despite the fact that the MP of the axoplasm was digested by the enzyme. Although intracisternal injection of H-ase into rabbits resulted in a disappearance of MP from the cells surrounding the cisterna magna, no gross behavioural changes were noticeable in the animals.

DISCUSSION

The present study leaves little doubt as to the presence of MP within the perikaryon, as well as in the dendrites and the axons of the mammalian central nervous system. Although the MP is attacked by purified testicular hyaluronidase, it remains to be determined whether the material is the same as the classical hyaluronic acid of the vitreous humour or umbilical cord. Some question exists as to specificity of the RINEHART–ABUL-HAJ iron stain for HA; the presence of HA can be established only after digestion with pure H-ase. Although the bulk of the MP was removed from the neuron with H-ase, a small amount still remained in the cytoplasm, whereas the nuclear material was unaffected. It cannot be stated that this enzyme-resistant material is not MP, or even HA, insofar as many mucopolysaccharides are not attacked by H-ase. The material staining with PAS, on the other hand, is not removed with H-ase and appears to be prevalent outside the neuron. The observations are in conformity with those of LUMSDEN (1958), who believed that MP existed in astrocytes in the form of PAS granules which migrated along the cellular processes into the tips from where they were discharged.

Insofar as the PAS-positive material is apparently not removed with H-ase, it probably is not HA. The blue-staining material, which is largely intraneuronal, is

in all likelihood HA. Preliminary biochemical findings suggest the presence of both an H-ase resistant and an H-ase susceptible acid mucopolysaccharide in mammalian brains.

Attempts to demonstrate the possible role of MP in the central nervous system have yielded largely negative results to date. Despite the fact that H-ase digestion appeared to remove the axoplasmic HA from lobster nerves, the electrical properties were normal. Intracisternal administration of H-ase, although resulting in partial removal of the MP from the rabbit brain, did not appear to produce any noticeable behavioural changes. It is conceivable that the techniques employed in this investigation were not refined enough to detect functional changes. Future studies of this nature are contemplated with the techniques of intracellular injection of the enzyme and electrical recording of single neurons.

The fact that embryonic neural tissue and regeneration axons contain an exceptionally large amount of HA is suggestive of its possible role in growth and development. Both of these preparations afford excellent opportunity for investigating the metabolic synthesis of HA within the nervous system, studies which are presently being pursued.

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SUMMARY

The presence of mucopolysaccharides has been histochemically demonstrated in the central nervous system of various mammalian species. Its presence in the central nervous system is ubiquitous, but is confined largely to the neurons and their processes, whereas the neuroglia are relatively free of the material. Insofar as the mucopolysaccharide is removable with hyaluronidase, it appears to contain hyaluronic acid.

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THE DETERMINATION OF COPPER IN SMALL QUANTITIES OF CEREBROSPINAL FLUID BY NEUTRON ACTIVATION ANALYSIS

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SEVERAL techniques—most of them spectrophotometric—have been used for investigating substances at very low concentration (a few p.p.m. or less) in biological fluids. These methods generally have a sensitivity limit in the microgram range and, owing to losses and contamination during the analytic procedures they are all liable to error. Neutron activation analysis seems to be a promising technique for overcoming these difficulties when trace substances in the submicrogram region are to be determined. BOWEN (1956, 1958) was able to determine manganese, copper and zinc in small quantities of blood by activation analysis.

In the present investigation neutron activation analysis was used in a study of the level of copper in small samples of the cerebrospinal fluid (CSF) from subjects presumed to be normal. Apart from sporadic determinations of copper in the numerous studies of Wilson's disease, only a few investigations of the copper levels in the CSF have been published (see CUMINGS, 1959, for references). So far as is known, only two studies (MUNCH-PETERSEN, 1950; D'ANDREA and CONGIU, 1952) on the copper levels in 'normal' subjects have been published.

METHODS

The spinal punctures were made in the lower lumbar region with a fine platinum needle, claimed by the manufacturer (KIFA) to be free from copper when examined by spectral analysis. With this fine needle lumbar punctures could be done in outpatients also. In order to prevent bending of the relatively flexible platinum needle during the procedure, it was introduced through a short, coarse conductor needle (ANTONI, 1923) of stainless steel. With the platinum needle in the subarachnoid space the CSF was allowed to drop freely into the test tubes. 8–10 ml were taken for the usual routine examinations, the last 5 ml being used to wash out the test tube, which had previously been cleaned with acetone, pure HCl and glass-distilled water.

The procedure of activation analysis used here is a modification and simplification of that described by BOWEN (1958). The technique has been developed at the Royal Institute of Technology in Stockholm (see WIDELL and WESTERMARK for a detailed description and critical analysis of the technique.) A few tenths of a ml of CSF were melted into small tubes of quartz. The samples along with the standards were irradiated for approximately 24 hr in a neutron flux of 2–3 $\times$ 10^{12} cm^{-2} sec^{-1}. The ^{63}Cu is activated to ^{64}Cu by the reaction: $^{63}Cu + (n, \gamma) \rightarrow {}^{64}Cu$. Chemical separation of the copper was performed after addition of carrier (LOVERIDGE and SMALES, 1957) by precipitation with salicylaldoxime. The chemical yield, determined by an electrolytic method (see WIDELL and WESTERMARK), was found to be about 50 per cent (variation 40–60 per cent). The gamma energy spectra as found by a NaI(Tl) scintillation detector were analysed with a multichannel pulse height analyser using the annihilation peak for the measurements (see Fig. 1).

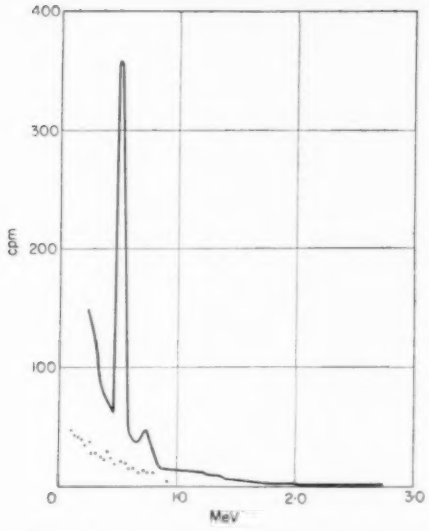

FIG. 1.—^{64}Cu in the CSF. Gamma energy spectrum of copper–salicylaldoxime precipitate with the annihilation peak at 0·51 MeV.

MATERIALS AND RESULTS

The determinations were made on eight patients diagnosed as psychoneurotics and with no objective evidence of organic disease. The CSF was examined for cells and total proteins and a mastix test was done. The results of all examinations were within normal limits, except in Case 1, whose CSF contained fresh red cells resulting from the puncture. This was probably the cause of the high copper content of the sample (Table 1). In the remaining patients no red blood cells were seen in the

TABLE 1.—THE COPPER CONTENT IN THE CSF OF EIGHT 'NORMAL' SUBJECTS

Case No.	Sample (g)	Cu in sample (g)	Copper content (g Cu/g sample)	Mean value (g Cu/g sample)
1	0·678	$2·5 \times 10^{-7}$	$7·4 \times 10^{-7}$	Only single sample
2*	0·631	$1·6 \times 10^{-7}$	$5·0 \times 10^{-7}$	Only single sample
3	0·249	$0·46 \times 10^{-7}$	$3·7 \times 10^{-7}$	$3·2 \times 10^{-7}$
	0·367	$0·4 \times 10^{-7}$	$2·7 \times 10^{-7}$	
4	0·360	$1·44 \times 10^{-7}$	$3·0 \times 10^{-7}$	$3·1 \times 10^{-7}$†
	0·273	$0·43 \times 10^{-7}$	$3·2 \times 10^{-7}$	
5	0·322	$0·42 \times 10^{-7}$	$2·6 \times 10^{-7}$	$2·4 \times 10^{-7}$
	0·244	$0·27 \times 10^{-7}$	$2·2 \times 10^{-7}$	
6	0·263	$0·68 \times 10^{-7}$	$4·4 \times 10^{-7}$	$3·8 \times 10^{-7}$
	0·405	$0·62 \times 10^{-7}$	$3·1 \times 10^{-7}$	
7	0·340	$0·43 \times 10^{-7}$	$3·0 \times 10^{-7}$	$3·3 \times 10^{-7}$
	0·340	$0·56 \times 10^{-7}$	$3·5 \times 10^{-7}$	
8	0·202	$0·61 \times 10^{-7}$	$6·4 \times 10^{-7}$	$5·9 \times 10^{-7}$
	0·187	$0·46 \times 10^{-7}$	$5·3 \times 10^{-7}$	

* A sample taken with the conventional needle contained about 25×10^{-7} g copper/g sample.
† Some increase in the copper value due to incomplete removal of ^{24}Na cannot be excluded.

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specimens. In all patients except Cases 1 and 2 double sample examinations were done. In Case 2 a special sample was taken with a conventional spinal puncture needle in order to decide whether or not the usual needles might be a source of copper contaminants. The copper value was about five times higher with this needle than with the platinum needle (cf. Table 1).

The chemical yield was experimentally determined in the first sample of Case 6 and in both the samples of Case 7 and 8. In Cases 1–5 a calculated mean value of 50 per cent for the method was used. After allowing for this the copper level in the CSF has been assigned a mean value of about 3×10^{-7} g/g (Table 1).

DISCUSSION

Investigations of trace substances in biological fluids require very sensitive analytical methods. The risk of contamination must be minimized and the chemical losses during the procedure estimated. The neutron activation method of analysis generally has a very high degree of sensitivity which makes it possible to determine submicrogram quantities of substances in contrast to the usual spectrometric methods which, in most instances, are accurate only in the microgram range. With the present method as little as 4×10^{-9} g copper can be determined (see WIDELL and WESTERMARK).

In analyses using neutron activation the risk of contamination is confined to a few initial steps of the procedure. After the sample has been irradiated and 'tagged' chemical sterility is no longer necessary. It is therefore extremely important to eliminate contamination as far as possible during the first operations. In order to achieve this, a special procedure for lumbar puncture was employed and the test tubes were scrupulously cleaned. Contamination of the CSF specimen with blood must be avoided (cf. Case 1, Table 1) otherwise abnormally high copper values may be recorded.

In order to investigate the contamination risk when needles made of chemically impure material are used, two lumbar punctures were made in one subject (Case 2, Table 1), one with the platinum needle and one with a conventional lumbar puncture needle. The copper value was about five times higher with the conventional needle than with the platinum needle indicating that the former is highly unsatisfactory for a quantitative determination of copper.

The chemical yield can be determined in different ways. In the present method a relatively large quantity of inactive copper (10–20 mg) as compared with the amount of copper in the sample was added as carrier. Therefore the chemical yield could be calculated with some certainty and was found to be about 50 per cent (cf. WIDELL and WESTERMARK).

Since submicrogram quantities of substances in biological fluids can be determined by neutron activation analysis, the samples can be small. This is of particular value when only small quantities are available for several tests as, for example, in the instance of the CSF. Thus the sample of CSF used for the determination of copper could be reduced to a few tenths of a ml (cf. Table 1) from the 2–6 ml for a single sample required by previous methods (AXTRUP, 1946; MUNCH-PETERSEN, 1950; D'ANDREA and CONGIU, 1952).

The high sodium and chloride concentration in biological fluids, however, presents special problems. Owing to the strong radiation from simultaneously activated

sodium and chlorine (^{24}Na, ^{38}Cl) special radiation shielding precautions are necessary and after radiation the sample must be 'aged' for some hour before the chemical analysis is commenced. The high activity of the ^{24}Na, and the fact that this element has a half-life similar to that of ^{64}Cu and also gives rise to an annihilation peak, can influence the gamma spectrometric results if ^{24}Na is not adequately separated during the chemical analysis. Methods for eliminating elements which may interfere with the gamma spectrometric measurements of copper are described by WIDELL and WESTERMARK.

With the present method a mean value of about 3×10^{-7} g copper/g CSF was found in seven 'normal' subjects. This figure is in agreement with that of D'ANDREA and CONGIU (1952) who reported a total CSF copper of about 27 μg per cent ($\approx 2{\cdot}7 \times 10^{-7}$ g/g) in 15 psychoneurotic patients using a spectrographic technique, but was higher than that reported by MUNCH-PETERSEN (1950), who with a spectrophotometric method found about 13 μg per cent ($\approx 1{\cdot}3 \times 10^{-7}$ g/g) in comparable material from 16 subjects.

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SUMMARY

The copper level in the cerebrospinal fluid was determined in eight 'normal' subjects by neutron activation analysis. The mean value was found to be about 3×10^{-7} g/g CSF. The method and the results are discussed. The necessity for scrupulous chemical sterility during the taking of the sample is stressed. The method has a very high sensitivity, a contamination risk confined to a few initial steps before irradiation, and permits accurate estimation of chemical losses during the procedure. For these reasons it may be considered a suitable method for studying small samples of biological fluids.

The radiation intensity resulting from the simultaneous activation of other substances in the specimen, notably ^{24}Na and ^{38}Cl, necessitates meticulous care in the handling of activated samples. It is pointed out that ^{24}Na may influence the final spectrometrical determination of ^{64}Cu if not chemically separated during the procedure.

Acknowledgements—This investigation was carried out in collaboration with the Division of Physical Chemistry at the Royal Institute of Technology, Stockholm. I am greatly indebted to tekn. lic. T. WESTERMARK and civ. ing. M. WIDELL for their help and critical discussions.

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AMINE OXIDASE AND 5-HYDROXYTRYPTAMINE IN DEVELOPING RAT BRAIN

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IN THE early development of mammalian brains there is a relatively short period of time during which the activities of several enzymes, though not all, increase from low levels to the higher levels characteristic of the adult. In the guinea pig this period, at least for frontal cerebral cortex, occupies the last trimester of gestation and is heralded by certain well defined cytological signs of differentiation, including the appearance of Nissl bodies and of cell processes (see FLEXNER, 1955, for review). In the rat, the corresponding period, preceded by similar cytological events, is from about the tenth to the twentieth days of postnatal life (SUGITA, 1918).

In view of the importance of monoamine oxidase for the metabolism of certain amines in brain (DAVISON, 1958; SJOERDSMA *et al.*, 1955) it was of interest to study the development of the enzyme's activity and to determine whether or not it is present in newborn rat brain. It has been suggested that one of its substrates, 5-hydroxytryptamine (serotonin) may be active at synapses (BRODIE and SHORE, 1957; UDENFRIEND *et al.*, 1957). Therefore, it appeared important to determine its concentration in the developing brain in relation to the development of amine oxidase.

MATERIALS AND METHODS

Animals. The rats used in these experiments were of Wistar strain. Most litters were born in the laboratory, but some were obtained from an outside supply. In both cases the births were known to within at least 18 hr and usually to within 10 hr. The day of birth was considered to be day 1. Litters used after the first 2 or 3 days of life were reduced to six animals. The number of brains used for each assay depended on their weight, so that several brains of very young animals were pooled, while after about 15 days of age, only one or two brains were used.

Preparation of tissue. Brains were removed in a standard, reproducible way by making one vertical cut just anterior to the cerebellum and another just posterior to the olfactory lobes; the optic tracts were severed, and the tissue was lifted out. The brains were quickly weighed, transferred to a glass homogenizer in ice, and homogenized with a motor driven Teflon pestle for 2 min in 4 times the volume of ice cold distilled water, or, in the case of serotonin analyses, 0·1 N-HCl. One ml of the water homogenate was transferred to a tared watchglass and dried at 40–50° to constant weight. This constituted the 'dry weight'.

Assay methods. For amine oxidase determinations, disappearance of tyramine was measured. Experimental flasks contained 1·0 ml of homogenate, 1·0 ml of sodium phosphate buffer, 0·2 M, pH 7·0, and 0·5 ml of tyramine hydrochloride (10 μmoles) previously neutralized to pH 7·0 and made up in 0·1 M-phosphate buffer. Control flasks received 0·5 ml of buffer instead of tyramine, or tyramine was added at the end of the experiment. When other substances were added, they were made up in phosphate buffer, and the buffer was correspondingly reduced. These conditions were chosen so that the disappearance of tyramine was as nearly as possible a linear function of the homogenate used (Fig. 1). Other concentrations of tissue gave nonlinear results. The flasks were shaken at room temperature in an atmosphere of oxygen for 3 min, and were incubated in an atmosphere of

oxygen for 30 min, at 37·3°. At the end of the experiment, 1·0 ml of incubation mixture was added to 1·0 ml of 0·3 M-$ZnSO_4$ and centrifuged, and the clear supernatant solution (pH ≈ 6) was diluted and assayed for residual tyramine by the method of UDENFRIEND and COOPER (1952). At least two estimations of total tyramine were made with each assay. Since these authors found that trichloroacetic acid precipitated variable amounts of tyramine with proteins, the recovery of tyramine from homogenates after $ZnSO_4$ precipitation was compared to the recovery of tyramine from water alone. In 24 observations, the recovery was from 94 to 107 per cent, with half of the observations falling between 98 and 102 per cent. When 0·15 μmoles of tyramine was carried through the procedure, a mean optical density (15 readings) of 0·309 was found, in agreement with the figure of UDENFRIEND and COOPER of 0·206 for 0·1 μmoles of tyramine.

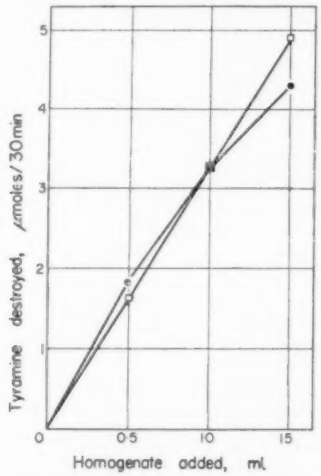

FIG. 1.—Dependence of tyramine disappearance on amount of homogenate present. □ = values expected from perfect linearity. ● = observed values.

Serotonin was extracted from the homogenates made in 0·1 M-HCl as described by UDENFRIEND, WEISSBACH and BRODIE (1958), and its fluorescence was examined at 550 mμ in 1 cm cells after activation at 295 mμ in a Farrand Optical Company spectrophotofluorometer.

Fluorescence was linear over the range studied. Since the emission is a function of temperature, the cells were kept in the light path for short periods only. Each reading was checked to insure that it coincided with a peak and that the activation for this wave length also exhibited a peak at 295–300 mμ.

Substrates. Tyramine was obtained as the hydrochloride from Distillation Products, 5-hydroxytryptamine as the creatinine sulphate complex from the California Foundation for Biochemical Research. The latter had a molecular extinction of $5{\cdot}66 \times 10^3$ at 275 mμ in 0·1 N-HCl at two concentrations. DPN was a product of Sigma Chemical Company. Reserpine* injections were given subcutaneously in doses of 2·5 or 5 μg/g body weight. It was dissolved in a few drops of glacial acetic acid and brought to pH 6 with water and NaOH.

Histochemistry. For demonstration of amine oxidase by reduction of a tetrazolium salt, the method recommended by GLENNER *et al.* (1957) was used, except that Na_2SO_4 was omitted. Sections of week-old and adult brains were cut in a cryostat, mounted on the same slide, briefly dried, and immersed in the medium without fixation. Incubation times were 15 min, 30 min, and 1 hr. The sections were photographed together under identical conditions.

RESULTS

When brains of a series of animals were studied the capacity of a 20% homogenate to destroy tyramine increased about three times on a wet weight and twice on a dry

* Reserpine was supplied through the courtesy of CIBA pharmaceutical company.

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weight basis during the first 20 days of life (Fig. 2). In both cases, the increase in activity during the first 10 days was very slight. On a wet weight basis, the rate of increase was then about three times as fast for the next 10 days, while on a dry weight basis the most rapid rise occurred during a 5 day period. Addition of DPN (10 μmoles) or semicarbazide (25 μmoles) to homogenates of 10 day-old or young adult brains did not alter the rate of disappearance. Neither of them interfered with the assay.

Frozen sections of unfixed brains from week-old and adult rats were examined

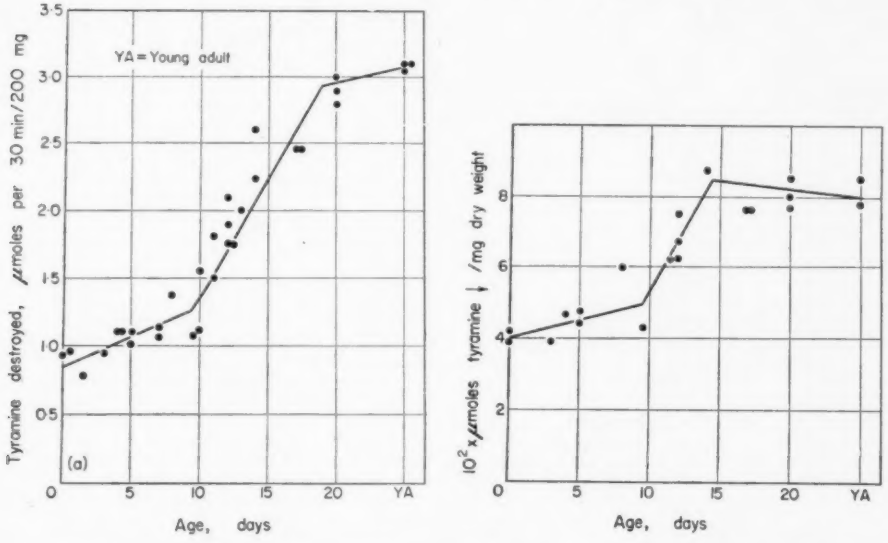

FIG. 2.—Disappearance of tyramine as a function of the age of the animal. Values are (a) per 200 mg fresh wt of tissue; (b) per mg dry wt of tissue.

for the distribution of amine oxidase by using the tetrazolium method of GLENNER *et al.* (1957) with tryptamine as substrate. Such sections showed diformazan granules distributed throughout the brains, although the precipitation was much less intense in the week-old tissue. Variations among areas, which were observed in the adult, were absent in the young brains (Fig. 3). Addition of DPN to the incubating medium resulted in somewhat denser precipitation of diformazan in both tissues, but the relative difference appeared to remain about the same.

Serotonin was present in 1-day-old rat brains at a level of approximately one-third that of adult brain and increased in linear fashion thereafter (Fig. 4).

Reserpine had a sedating effect on animals of all ages studied: the youngest was 6 days old. The doses used were 2·5 and 5 μg/g of body weight. A single dose produced a profound effect that lasted for 3 days. The animals became drowsy and cool, and they failed to move about and to suckle. The magnitude of the effect on weight gain can be seen from Fig. 5, where the suppression of growth from two separate doses of reserpine is plotted.

Brains taken from treated animals at 18–20 days of age contained levels of amine oxidase activity that were 65 to 75 per cent of those from littermates of the same age.

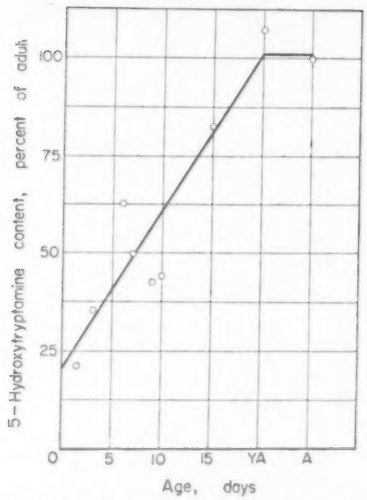

FIG. 4.—Serotonin concentrations in developing brain as percentages of adult concentrations. The adult value is the mean of three determinations which differed from the mean by 7 per cent or less.

The serotonin levels reported here are plotted as percentage of adult levels, because it has been reported (UDENFRIEND *et al.* 1958) that serotonin added to brain and subsequently extracted yields higher values than when extracted from aqueous media alone. The magnitude of this effect may well vary with the changes in brain composition that take place with age. However, an approximation to the absolute quantities involved may be gained if this effect is assumed to be small. Then, if one takes the figures of CORREALE (1956) and UDENFRIEND *et al.* (1957) for whole brains, the adult contains 0·4 μg/g of serotonin. If the ratio of newborn to adult concentration is the same for whole brain as for brain as removed here, the newborn would contain about a third, or 0·13 μg/g serotonin. This means that per newborn brain there is only about 0·03 μg serotonin present, while the adult brain would contain about 0·6 μg. Hence the serotonin per brain increases by a factor of roughly twenty during this period.

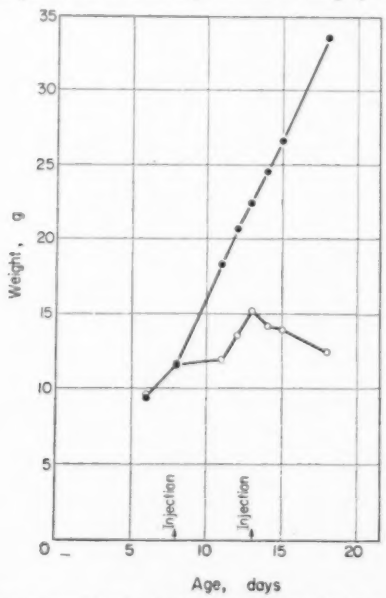

FIG. 5.—Typical effect on growth of a single dose of reserpine (5 μg/g) given subcutaneously. ● = growth of littermate given solvent alone. ○ = growth of animal given reserpine.

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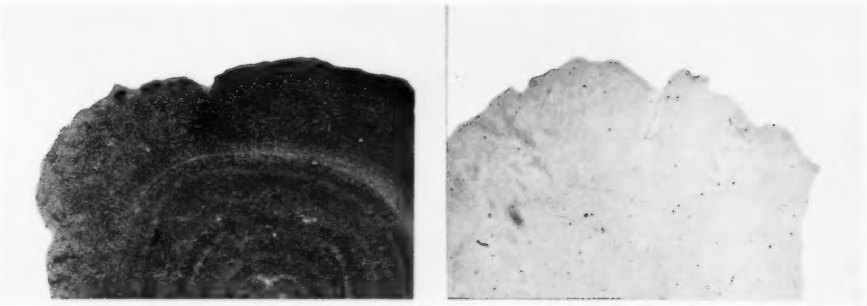

FIG. 3.—Comparable portions of sections of adult brain (left) and week-old brains (right) treated histochemically for amine oxidase activity. Sections were incubated on the same slide and treated identically in the photographic process. Note the homogeneous distribution of precipitate in the young brain as compared to the adult.

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Such animals were always smaller than their littermates: body and organ weights, including brain weights, were 50–80 per cent of those of controls, depending on the timing and frequency of the dose and upon the organ.

DISCUSSION

These results add another enzyme to the group of those whose activity in the central nervous system increases during postnatal development in the rat. In general, the activities have been expressed per unit wet or dry weight of tissue, and if one assumes that they reflect actual increases in protein, it is clear that in order for a balance sheet to be maintained, some cellular components must be decreasing in amount per unit weight during this period. Concentrations of some small molecules may diminish in this period: BURTON (1957) found that the DPN content of rat brains, when calculated on a trichloroacetic acid-insoluble dry weight basis, diminished with age. However, the major substance known to decrease, at least for the corresponding growth period in the guinea pig, is desoxyribosenucleic acid. The data of FLEXNER and FLEXNER (1951) show that in guinea pig cortex, the DNA fell by a factor of two during the last trimester of gestation. The histological appearance of newborn rat brain as compared to the adult strongly suggests that a similar relationship holds here, since the number of cell nuclei per unit area decreases markedly with the growth of cytoplasm, including the cell processes.

But although many enzymes increase in activity, the rates at which they do so differ. The results reported here put amine oxidase into the larger group, which includes succinic dehydrogenase and adenosine triphosphatase (POTTER, SCHNEIDER and LIEBL, 1945), phosphoglucomutase (SHAPIRO and WERTHEIMER, 1943), and cytochrome oxidase and aldolase (HAMBURGH and FLEXNER, 1957).

A difference, therefore, exists between these enzymes and cholinesterase and diphosphopyridine nucleotidase (BURTON, 1957), since the activities of the latter two increase rapidly during the first week of postnatal life as well as in the second. While perhaps not too much emphasis can be laid on this distinction, it may indicate that different factors control the rates of formation of different enzymes. For example, it has been reported (CRAIN, 1952) that continuous electrical activity is recorded from rat's cortex only after the first week of life. Possibly, then, enzymes that increase most rapidly after this period do so in response to increased physiological activity.

Another possible factor influencing the rate of development of activity is the cytological localization of the enzyme. Amine oxidase, for example, is found chiefly in the mitochondrial fraction (DAVISON, 1958) and the development of its activity parallels that of succinic dehydrogenase and cytochrome oxidase. Therefore it is possible that several enzymes may be controlled together, and that increases in activity are secondary to the problem of development of cell organelles.

Yet another factor that may influence the rate of development is substrate concentration. Experiments with other systems have indicated generally only transient effects of altering the substrate level upon the enzyme activity during development (GORDON and RODER, 1953; ROEDER, 1957). However, in these cases it was likely that the substrate levels remained above normal for only a short period. In the present experiment, a device was sought to reduce the level of the substrate during development, and to use a substance that would produce a prolonged effect. While the results did show a reduction in amine oxidase activity of animals so treated, they

cannot be interpreted until the nutritional effect of the reserpine is separated from its effect upon the levels of a number of brain substrates.

SUMMARY

(1) Amine oxidase activity was estimated by the disappearance of tyramine from homogenates of 1–40-day-old rat brains, removed so that cerebellum and olfactory lobes were excluded. On the basis of the wet weight of tissue, activity increased during the first 20 days by a factor of three. On a dry weight basis the increase was two-fold. In both cases the rate of increase was very slow during the first 10 days.

(2) By a histochemical method, the oxidase was found to be widely distributed in brains of 8-day-old and adult rats. The 8-day-old rats appeared to have much less activity, and to lack the local differences prominent in the adult.

(3) Serotonin was present in the brains of newborn rats at about a third of the concentration in adult brain, and increased in a linear fashion thereafter.

(4) Reserpine produced profound sedation in animals during this growth period when given in single doses of 2·5 to 5 μg/g. A single dose was sufficient to suppress weight gain almost entirely for 3 days. Brains of animals treated twice or more during the first 20 days of life contained levels of amine oxidase activity from 65 to 75 per cent of those of controls when assayed at 20 days of age.

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THE ELECTROPHORETIC DISTRIBUTION OF THE LIPOPROTEINS OF THE SUPERNATANT FRACTION OF RAT WHOLE BRAIN

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AT THE present time, knowledge of the structure and function of the lipoproteins of the central nervous system is scanty. FOLCH and LEES (1951) have described proteolipids which are extractible in lipid solvents, and LEBARON and FOLCH (1959) have made an investigation of the conditions governing the complete extraction of proteins (and some lipoproteins) from nervous tissue. HOFMANN and SCHINKO (1956) have briefly reported experiments on the staining of lipids after the paper electrophoretic separation of the soluble lipoproteins of human brain.

In the present paper, a composite method is described by means of which the distribution of soluble protein (i.e. protein extractible in aqueous solution at pH 7 or 8 in the presence of salts at concentrations less than 1 N) and associated lipid was determined by paper electrophoresis. The rate of uptake of labelled phosphorus into the lipoprotein lipids was also investigated.

Briefly, the studies comprised the administration of labelled orthophosphate to rats by subarachnoid injection, and the extraction of the soluble proteins of the brain, after allowing different lengths of time for the isotope, ^{32}P, to be taken up into the tissue.

After paper electrophoresis of the extracts, the relative amounts of protein, phospholipid and cholesterol present in the lipoprotein fractions were evaluated. The autoradiographs and activity counts for lipid ^{32}P at different times after injection gave some information about the process of labelling.

METHODS

The following operations were carried out in the order in which they are presented. Most of these were adaptations of existing experimental methods and do not require detailed treatment here.

Preparation of an extract of labelled lipoproteins from whole rat brain. In each of five experiments, two albino rats (Wistar strain) were each given an injection of 70 μc of [^{32}P] orthophosphate in isotonic saline (pH 7) into the subarachnoid space. The first pair was left for 1·5 hr (Experiment 1), the second for 3 hr (Experiment 2), the third for 12 hr (Experiment 3), the fourth for 36 hr (Experiment 4) and the fifth for 60 hr (Experiment 5) before the animals were killed and the brains removed. The rats were anaesthetised with ether and killed by cutting the jugular veins. The brain was perfused with 2 ml of distilled water through the internal carotid arteries. The brains were removed, rinsed and homogenized in 3·5 ml of the buffer used for the electrophoresis. The water-clear supernatant fluid (3·5 ml) resulting from centrifugation of the homogenate at 12,000 g for 20 min was immediately used for electrophoresis. Portions of the extracts were kept for the determination of protein nitrogen, the lipid phosphorus of the material precipitable with 5% sulphosalicyclic acid, and the activity of this labelled lipid. The acid-soluble phosphorus and its count rate were also determined.

Paper electrophoresis of extract of soluble protein from rat brain. The method of paper electrophoresis has already been described (ROBERTSON, 1960). Before use, the electrophoresis papers were

extracted with chloroform–methanol (2:1) by hot reflux for 20 days, dried and soaked in the electrophoresis buffer for at least 12 hr. In each experiment, 0·12 ml of extract was used on each of 30 electrophoresis strips. The soluble proteins of the first four experiments separated into 9 fractions, including the material remaining at the point of application, on a total of 120 electrophoresis strips. The 60-hr extract was not used for paper electrophoresis. These fractions were stained for protein with bromophenol blue by the method of JENCKS, JETTON and DURRUM (1954) after fixation of the proteins on the paper with 5% sulphosalicylic acid. The boundary lines between the stained fractions were marked out in pencil and the air-dried strips kept in closed containers until required.

Estimation of electrophoretic protein. The 30 developed electrophoresis strips in each of the first four experiments were divided into three groups of 10. One of these groups was used for the determination of the amounts of cholesterol and protein which were present in the electrophoretic fractions; a second group was used for the extraction of lipid from the fractions, the determination of count rates of this lipid and its phosphorus content, and the third group for the chromatography of the lipid from the fractions.

For the estimation of cholesterol, the 10 strips were divided up along the pencil lines between the electrophoretic fractions and similar paper segments placed together. Cholesterol was extracted from the 9 batches of paper segments with diethyl ether and estimated by the method of ZLATKIS, ZAK and BOYLE (1953) using ferric chloride in concentrated sulphuric acid. After the removal of cholesterol, bromophenol blue was eluted from the paper segments with weak alkali, and the optical density of the dye read at 3600 Å. The amounts of protein corresponding to the absorbance of the dye were found from calibration curves.

Extraction of labelled lipids from the electrophoretic fractions. The 10 electrophoresis strips were divided up as before and the like segments pooled. The 9 batches of papers were extracted overnight with chloroform–methanol (2:1) at 40°. Non-lipid phosphorus was removed by the method of FOLCH, LEES and SLOANE-STANLEY (1957) in the manner recommended for labelled phosphatides. Count rates were determined on each of the 9 lipid fractions in organic solvent and the figures corrected for radioactive decay and background. The lipid was ashed by heating with perchloric acid and the lipid phosphorus estimated by the method of CHEN, TORIBA and WARNER (1956) using an ascorbic acid–ammonium molybdate reagent to develop a blue colour. This colorimetric method is claimed by the authors to be eight times more sensitive than that of FISKE and SUBBAROW (1925) and the sensitivity was further increased threefold by extraction of the blue pigment into 2 ml of *iso*-butanol–diethyl ether (1:2). The colour was stable for some hours in this solvent and the absorbance was read at 8200 Å against a distilled water blank.

Chromatography of the labelled lipid from the electrophoretic fractions. The last 10 electrophoretic strips from each of the four experiments were divided up and the lipids extracted from each of the segment pools. Chromatography of the four batches of 9 lipid extracts was carried out on silica-impregnated paper by the method of LEA, RHODES and STOLL (1955), about 35 μg of phospholipid were applied to each chromatogram. The developed chromatograms were left in contact with Kodak Medical X-Ray film for 4 weeks, and the resulting autoradiograms developed and photographed.

Chromatograms and autoradiographs were also made from the protein-bound lipid of Experiment 5 (60 hr after injection of [^{32}P] orthophosphate into rat brain). Heavy labelling of 5 phospholipids was shown by the autoradiograph (Fig. 1, right) although only three or four lipids were revealed by staining the chromatogram with Luxol Blue, a dye with some affinity for phosphatides. An autoradiograph for the lipids from the original extract of rat brain of Experiment 4 (36 hr exchange with ^{32}P) was also made (Fig. 2, centre). Autoradiographs of mitochondrial phospholipids (right) and microsomal phospholipids (left) after the same exchange time of 36 hr, are included for comparison. No quantitative estimations of the amounts of the individual phospholipids which were separated on the chromatograms were made.

From the figures, it may be seen that two of the chromatographically separated phospholipids of the lipoprotein of the rat brain extracts are much more heavily labelled than the others (spots 3 and 4), and are generally well separated from one another. Most of the lipid from the acid-precipitable fraction of extract from Experiment 5 (60 hr exchange) was chromatographed; the spots were lightly stained; spots 3 and 4 cut out, and the collected paper areas pooled into two lots and the lipid extracted. The lipid from spot 3 was identified as 'lecithin' and that from spot 4 as 'cephalin' by the following criteria.

One spot occupied the R_F position given by LEA *et al.* (1955) for lecithin (spot 3; R_F value, 0·63)

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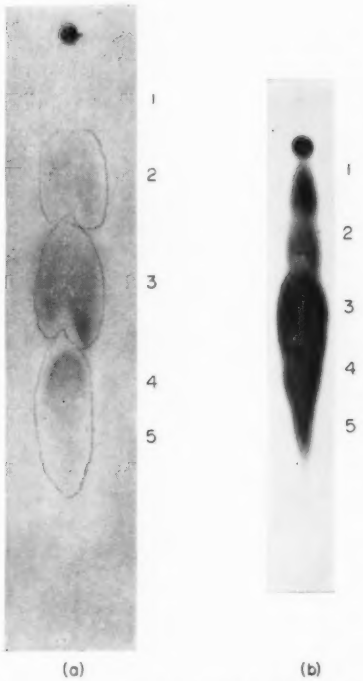

FIG. 1.—(a) Chromatographic separation of the ^{32}P-labelled phospholipids of the soluble (supernatant) lipoproteins of the rat brain 60 hr after injection of [^{32}P] phosphate. (b) The corresponding autoradiograph (on a smaller scale).

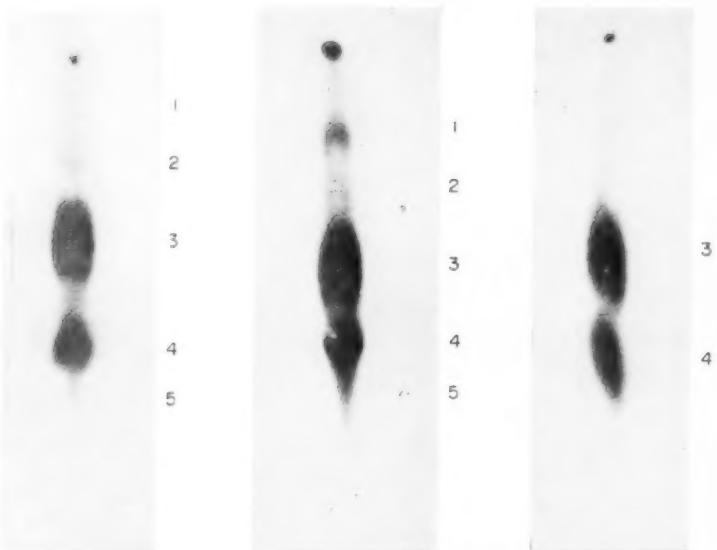

FIG. 2.—Autoradiographs of chromatographic separations of ^{32}P-labelled phospholipid from the lipoproteins of the supernatant (centre), the mitochondrial (right) and the microsomal (left) fraction of rat whole brain.

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and the other for cephalin (spot 4; R_F value, 0·80). The autoradiographs published by MARINETTI, WITTER and STOTZ (1957) which were made from the ^{32}P-labelled lipids of rat tissue, including brain, resembled those of the present writer very closely and were made by the method of LEA *et al.* (1955). The autoradiograph shown by MARINETTI *et al.* (1957) for the lipids of brain (intraperitoneal injection was used) showed only two spots identified by the authors as being lecithin and cephalin. This autoradiograph resembled that shown in Fig. 2 (right).

The lipid from both chromatographic spots was hydrolysed for 3 hr with 6 N-methanolic hydrochloric acid. After removal of the methanol and the acid under reduced pressure, the residue was partitioned between ether and water. The aqueous phase was separated and dried. Reinecke salt, when added to some of the hydrolysate of spot 3, in aqueous solution, caused the formation of a red precipitate. Negligible amounts of such precipitate were given by samples of the hydrolysate of spot 4.

The hydrolysis products of both chromatographic spots were separated by the paper chromatographic method of MEREDITH and SAMMONS (1952). Choline chloride, ethanolamine and serine were used as standards for comparisons of R_F values and colour reactions (ninhydrin for serine and ethanolamine; the precipitation of phosphomolybdic acid for choline). The hydrolysate from spot 3 was found to contain choline but no serine or ethanolamine. The hydrolysate from spot 4 contained ethanolamine, but neither serine nor any other ninhydrin-reacting compound could be detected on the chromatograms.

Estimation of nucleic acid in the electrophoretic fractions of soluble protein from rat brain. Paper segments from the divided electrophoresis strips were retained after the extraction of lipid, and examined for the presence of nucleic acid by the method of LOGAN, MANNELL and ROSSITER (1952). The segments were hydrolysed in 5% trichloroacetic acid for 15 min at 90° and the filtrate examined for absorption at 2685 Å. The blank was an hydrolysate of 100 cm² of the electrophoresis paper and standards for calibration of the absorption readings were made from hydrolysed deoxyribonucleic acid (J. Light and Co.).

RESULTS AND DISCUSSION

The distribution of protein, lipid and nucleic acid in the electrophoretic fractions of extracts of whole rat brain. The tissue extracts contained (mean values for 5 extracts) 13·45 mg of soluble protein, 1125 μg of protein-bound phospholipid and 375 μg of protein-bound cholesterol per ml. Approximately 11 per cent of the solids of the acid-precipitable material of the extracts was lipid; the rest being mainly protein. Very small amounts of protein-bound nucleic acid were found in the extracts, amounting to only 21 μg per ml. The protein-bound cholesterol was exactly one-third of the phospholipid in quantity. The lipids attached to the protein in the extracts showed signs of having unsaturated aldehydic groups; e.g. decolorized fuchsin-sulphurous acid regained its colour in the presence of this lipid extracted with ethanol.

The distribution of the phospholipids among the fractions did not follow that of the proteins (Table 1), but an unknown amount of free protein was present. In the last two fractions (7 and 8) cholesterol showed a large increase relative to phospholipid. It was not found possible to stain the lipoprotein on the electrophoresis strips clearly enough for photography, but the author was satisfied that the lipid formed discrete fractions in close association with the proteins. All the protein fractions were clearly seen to have some attached lipid.

The uptake of labelled phosphate into the lipid of the electrophoretic fractions of rat brain soluble protein. Table 2 contains the figures for the relative specific activities of the four sets of 9 lipid extracts from the electrophoresis experiments. After the short (1·5 hr) period of exchange of ^{32}P in rat brain, the slow-running fractions (0 and 1) and the rapidly-running fraction 8, were found to have three times the specific activity ratios of the others (2–7). The rate of increase of activity of the lipid from the different electrophoretic fractions with increasing time of exchange of isotope in the rat brains,

TABLE 1.—VALUES FOR THE DISTRIBUTION OF PROTEIN, PHOSPHOLIPIDS, CHOLESTEROL AND NUCLEIC ACID AMONG NINE ELECTROPHORETIC FRACTIONS SEPARATED FROM EXTRACTS OF RAT WHOLE BRAIN

The amounts given are expressed in μg for one electrophoresis strip (i.e. 0·12 ml of extract). S.E.M. = Standard error of the mean

Electrophoretic fraction	Mean migration distance (cm)	Protein (dye elution) (μg)	± S.E.M. (ten strips)	Phospholipid (lipid P × 25) (μg)	± S.E.M. (four experiments)	Cholesterol (μg)	Nucleic acid (μg)
Origin 0	—	300	—	78·0	—	24·0	0·8
1	1·6	115	7·8	7·6	0·60	2·2	trace
2	2·5	139	9·0	5·6	0·40	1·8	0·2
3	3·4	239	15·5	6·9	0·31	2·4	0·4
4	4·4	211	11·5	7·5	0·22	3·2	0·3
5	5·0	249	15·5	11·6	0·78	3·8	trace
6	6·5	254	8·5	10·7	0·99	2·6	0·2
7	7·5	225	23·5	3·4	0·38	3·0	0·3
8	10·0	99	9·5	2·7	0·21	2·4	trace
Serum albumin 0·05 ml	6·0						

TABLE 2.—THE SPECIFIC ACTIVITY RATIOS (×100) OF PHOSPHOLIPID PHOSPHORUS FROM PAPER ELECTROPHORETIC FRACTIONS OF ^{32}P-LABELLED EXTRACT OF RAT WHOLE BRAIN

The specific activity ratio is defined as:

$$\frac{\text{Counts/min per mg lipid P in 10 ml of solution}}{\text{Counts/min per mg acid-sol. P in 10 ml solution}}$$

Electrophoretic fraction	Experiment 1 1·5 hour exchange period	Experiment 2 3 hour exchange period	Experiment 3 12 hour exchange period	Experiment 4 36 hour exchange period
Origin 0	19·2	32·0	40·7	34·4
1	20·2	74·0	91·0	111·0
2	7·6	26·0	38·0	37·0
3	7·2	23·5	28·8	36·4
4	6·9	21·4	35·5	44·3
5	6·1	24·4	31·4	39·4
6	5·4	24·0	24·0	27·0
7	9·4	14·9	30·6	37·0
8	13·7	18·1	25·2	26·0
Lipid phosphorus of 0·12 ml	68·5	38·8	50·0	65·0

was not the same. There are two possible explanations for this; either different types of phospholipid with different turnover rates were present in the different lipoprotein fractions, or the fractions had the same or a similar phospholipid content but the individual phospholipids became labelled in a different time sequence.

The pattern of the chromatographic separation of phospholipids from each of the electrophoretic fractions was found to be qualitatively the same, but it is not known

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whether the relative amounts of the individual lipids was different in each fraction. The activity patterns revealed by autoradiography of the chromatograms clarified the significance of the specific activity ratios given in Table 2. In the autoradiograph of lipid from Experiment 5 (Fig. 1), a phospholipid situated just below the point of application of the mixture to the paper showed strong activity, although no corresponding spot was visible on the chromatogram itself. A possible explanation of this might be that the actual amount of the phospholipid was very small, but the radioactivity was high.

When autoradiographs were made from the chromatograms of the electrophoretic lipid of Experiment 1 (1·5 hr exchange of ^{32}P in rat brain) all of the activity patterns showed two patches only, which were faint and corresponded to the positions of lecithin and cephalin. All nine autoradiographs presented an appearance similar to that of mitochondrial phospholipid (36-hr exchange) shown in Fig. 2, right. The autoradiographic pattern for lipid from electrophoretic fraction 1 began to show areas of activity corresponding to the chromatographic spots 1 and 2 after the 12 hr period of exchange of ^{32}P in rat brain. The presence of this activity in the chromatographic spots became more marked with increasing exchange time as the lipid from fraction 1 took up more labelling. The high starting value of the specific activity ratio of the lipid from electrophoretic fraction 1, relative to the others, may be taken as evidence that the chromatographic lipids 1 and 2 have very rapid turnover rates.

The relatively high initial values of the specific activity ratios of lipid from the origin and fraction 8 are less easy to account for, as no activity could be detected in positions other than for lecithin and cephalin. It is possible that the chromatographic spots for these two phospholipid types conceal or merge with others in the same positions, which have active turnover rates. The tailing seen below the autoradiographic patch for cephalin in Fig. 2 (centre), and designated chromatographic spot 5, was actually an incompletely separated, minor phospholipid. Using a two-dimensional system of paper chromatography, MARINETTI *et al.* (1957) were able to produce an autoradiograph of ^{32}P-labelled phospholipids of heart showing at least fifteen different areas of activity.

The specific activity ratios of the electrophoretic lipid from fractions 2 to 7 were very similar, and it seems likely that they originated from the labelling of lecithin and cephalin only.

It has been shown by ANSELL and DOHMEN (1957) that the rate of uptake of labelled phosphorus into the different phosphatides of rat brain is not the same. CHANGUS, CHAIKOFF and RUBEN (1938) were able to show that the peak of ^{32}P incorporation into rat brain lipids is reached in 8 days. This finding was confirmed by DAVISON and DOBBING (1959) who further reported that the degree of labelling falls to two-thirds of the original value after 70 days. DAWSON (1954) found that in cat brain 7 days after the administration of ^{32}P the high activity of glycerylphosphoryl choline from lecithin and glycerylphosphoryl ethanolamine from cephalin were only exceeded among the partial hydrolysis products of labelled phosphatides of rat brain by inositide phosphate from diphosphoinositide. According to the autoradiographs of MARINETTI *et al.* (1957) the chromatographic spot 1 of the present writer may be an inositol phosphate. The topic of lipid metabolism in the central nervous system has been reviewed by ROSSITER (1957).

It is likely that the lipoproteins which are readily extracted in simple saline or weak

alkaline solution of low molarity from brain tissue, may form a pool of 'solubilized' phospholipid which can be drawn upon for the formation of the less soluble structural lipoproteins. It is also possible that these soluble lipoproteins can donate phospholipid as a secondary source of energy, under certain circumstances. ABOOD and GEIGER (1955) have published figures for the percentage decrease in the amount of phospholipid phosphorus of the acid-precipitable fraction of extracts of cat cortex, in the absence of blood glucose. This was 33 per cent in one experiment and 63 per cent in another. For microsomal phospholipid the values were 22 per cent and 64 per cent and for mitochondrial phospholipid they were 3 per cent and 5 per cent. Additional evidence that the phospholipids of the acid-precipitable (lipoprotein) fraction in brain have an active turnover rate relative to those of the less soluble, structural lipoproteins, is supplied by experiments on the uptake of ^{32}P into the particulate fractions of rat

TABLE 3.—THE UPTAKE OF [^{32}P] ORTHOPHOSPHATE INTO THE LIPIDS OF SUBCELLULAR FRACTIONS OF THE RAT BRAIN

Specific activity ratio ($\times 100$) of lipid phosphorus relative to acid-soluble phosphorus at different times after subarachnoid injection of [^{32}P] orthophosphate. Each animal (80–100 g) received 100 μc ^{32}P in 0·40 mg NaH_2PO_4 carrier. Four rats were used in each of the three experiments

Exchange period	Specific activity ratio $\times 100$				
	Nuclei	Mitochondria	Microsomes	Supernatant	Cell debris (myelin)
1 hr	14·4	22·4	37·6	37·7	11·0
60 hr	48·7	60·5	59·5	59·5	52·5
11 days	81·0	131·0	95·0	80·0	75·0

brain tissue. After subarachnoid injection of labelled phosphorus into rat brain and allowing an exchange period to elapse, a tissue particulate suspension was made by the method of BRODY and BAIN (1952). Lipid was extracted from the acid-insoluble material of the supernatant fraction, as well as from mitochondria, microsomes, nuclei and the cell debris. The specific activity ratios relative to the acid-soluble phosphorus of each fraction are given in Table 3. After an exchange period of 1 hr the specific activity ratios of microsomes and supernatant were about 37·7, while the specific activity ratio of cell debris, which in the case of brain consists largely of myelinated structures, was only 11. The phospholipids of the supernatant acid-precipitable fraction, were found to take up ^{32}P more rapidly than those of the other particulate fractions, but also to lose activity more rapidly with time.

In the hands of the present writer, the methods described above were found to be more precise and informative than the conventional methods of showing lipid and carbohydrate on electrophoretic separations of brain proteins. The continued usefulness of paper electrophoresis in such studies as those described in the present paper should be stressed.

SUMMARY

(1) The electrophoretic distribution of protein, cholesterol, phospholipids and nucleic acid has been examined in extracts of the soluble lipoprotein of rat whole brain.

(2) The radioactivity of the phospholipid from the electrophoretically separated

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lipoproteins of rat brain, after labelling with ^{32}P-orthophosphate, has been examined. Specific activity ratios of the lipid from the electrophoretic fractions and autoradiographs of chromatographic separations of the individual phospholipids have been used to form a picture of the process of labelling of these lipids *in vivo*.

(3) The significance of the tracer experiments described in the present paper on the labelling of the lipids of soluble lipoproteins, is discussed.

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STUDIES ON CONJUGATED LIPIDS—VIII

ISOLATION OF A DIHYDROCEREBROSIDE FROM HUMAN BRAIN

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KLENK (1925, 1926, 1927) originally isolated both nervone and hydroxynervone from a fraction of a diethyl ether or petroleum ether extract of human brain. We have investigated this lipid fraction with the intent of isolating nervone and hydroxynervone in a pure state. It was expected that unknown substances might be isolated during purification of these fractions. A complete fractionation of ether-extractable cerebrosides was attempted, human brain being used as a source. A new cerebroside with an iodine number of zero was isolated.

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EXPERIMENTAL

Analytical methods. Iodine number was determined by the micromethod of YASUDA (1931), nitrogen by the micro-Dumas procedure (OCHIAI, TUDA and SAKAMOTO, 1948) and phosphorus by the method of LIEB (1924). The new cerebroside was isolated as shown in Fig. 1.

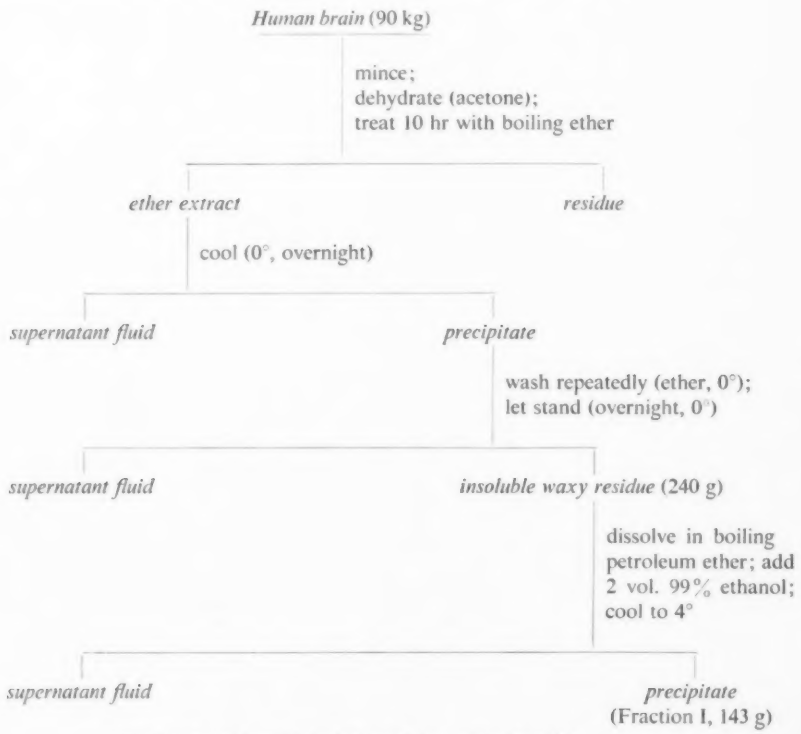

FIG. 1.—Procedure for isolation of the new cerebroside

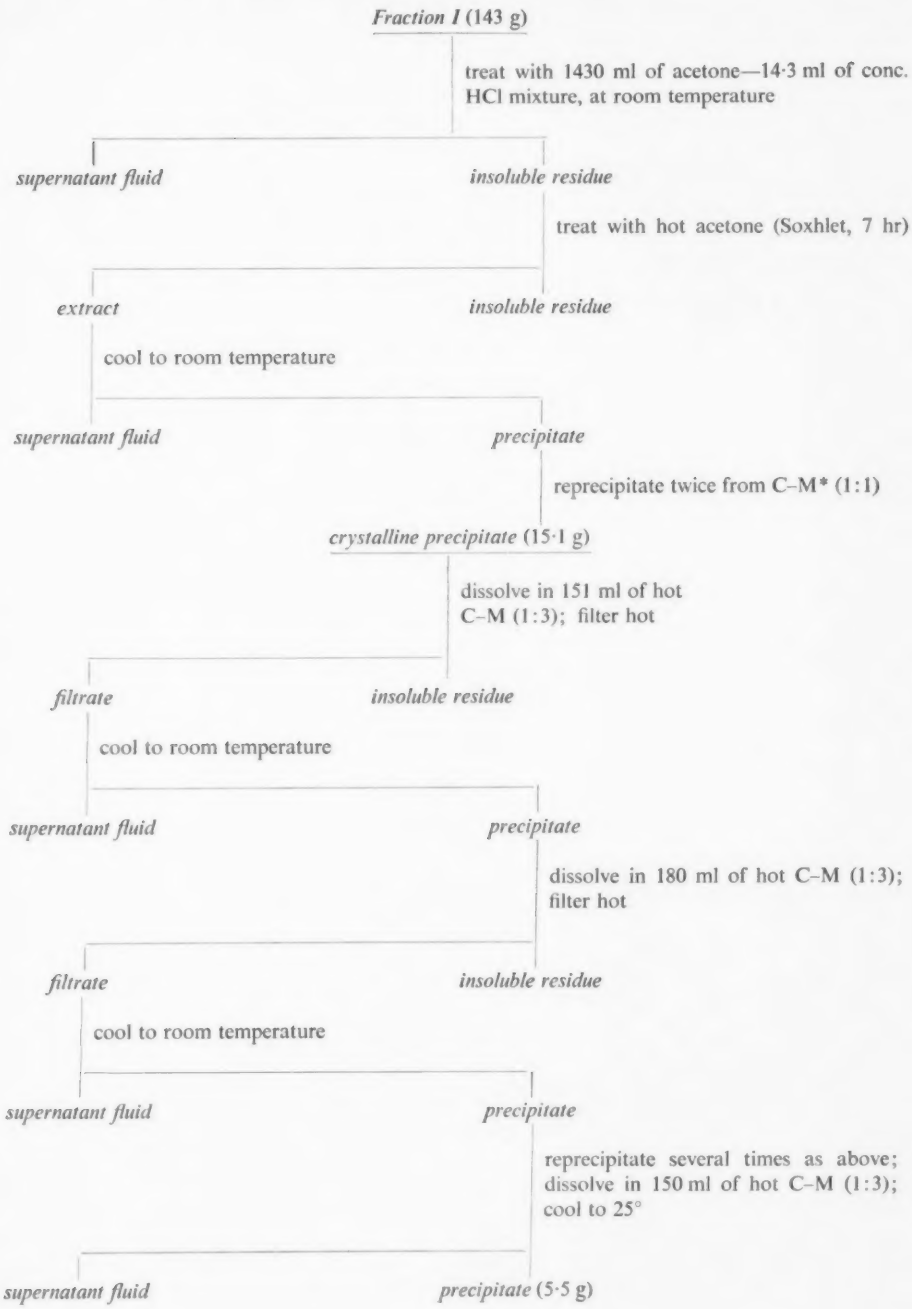
Fraction I (143 g)
treat with 1430 ml of acetone—14·3 ml of conc. HCl mixture, at room temperature
supernatant fluid
insoluble residue
treat with hot acetone (Soxhlet, 7 hr)
extract
insoluble residue
cool to room temperature
supernatant fluid
precipitate
reprecipitate twice from C–M* (1:1)
crystalline precipitate (15·1 g)
dissolve in 151 ml of hot C–M (1:3); filter hot
filtrate
insoluble residue
cool to room temperature
supernatant fluid
precipitate
dissolve in 180 ml of hot C–M (1:3); filter hot
filtrate
insoluble residue
cool to room temperature
supernatant fluid
precipitate
reprecipitate several times as above; dissolve in 150 ml of hot C–M (1:3); cool to 25°
supernatant fluid
precipitate (5·5 g)

FIG 1. (*contd.*)

* The abbreviation used is : C–M, Chloroform–Methanol.

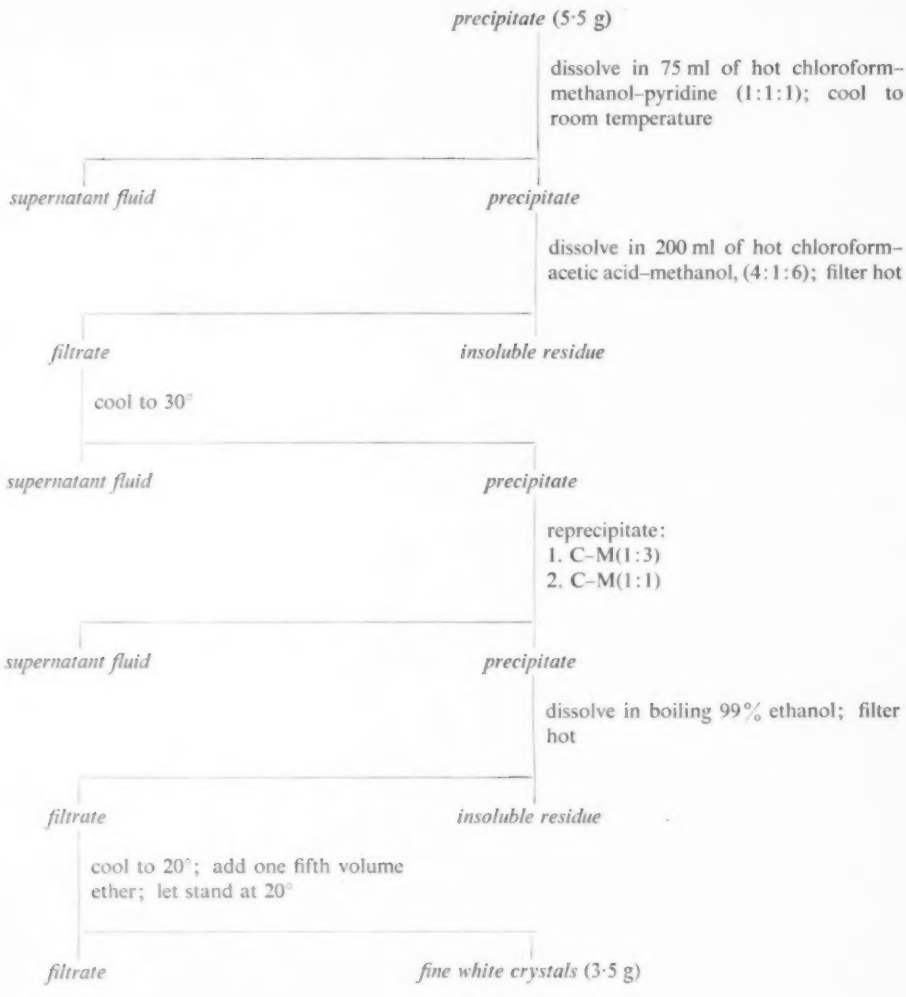

FIG. 1. (*Contd.*)

Methanolysis and separation of components. 3·4 g of the crystalline cerebroside (Fig. 1) were added to a solution of 3·8 ml of concentrated H_2SO_4 in 78 ml of absolute methanol, and the mixture was heated at 100° for 5½ hr and left overnight at 0°. The precipitate (fatty acid esters) was separated. Traces of ester in the filtrate were extracted with petroleum ether and added to the former. The remaining solution was concentrated and made neutral to phenolphthalein, and the precipitate was removed by filtration. The filtrate was made acid to litmus, concentrated, and made strongly alkaline with methanolic KOH. The ether extract of this solution, after washing with water, drying over sodium sulphate, and removal of solvent, yielded a white, non-hygroscopic base. A syrupy sugar fraction was obtained from the ether-extracted aqueous phase.

Analyses. The new cerebroside (3·5 g) melted at 196–8°. The iodine number was zero. Tests for unsaturation with $C(NO_2)_4$ and with $KMnO_4$ were negative. $[\alpha]_D^{23}$ in pyridine was +10. Anal. Calc. for $C_{48}H_{95}O_9N$ (Mol. Wt. 830) C, 69·44;

H, 11·53; N, 1·68%. Found: C, 69·11; H, 11·35; N, 1·66%. Hexose was determined by the method of HAGEDORN and JENSEN (1923) after hydrolysis with 20% H_2SO_4–ethanol (1:1) for 1 hr and subsequent extraction of fatty acid and sphingosine with ether (BRAND and SPERRY, 1941; ZIKKEN KAGAKU KOHZA, 1957). (Calculated for hydroxylignoceryl cerebroside: 21·70%. Found: 21·47%. The free fatty acid (1·6 gm) was prepared by saponification of the ester fraction (1·7 gm.) and crystallized from ethanol. Anal. Calc. for hydroxylignoceric acid, $C_{24}H_{48}O_3$ (Mol. Wt. 384·6) C, 74·94; H, 12·58%. Found: C, 74·80; H, 12·41%. The melting point was 100–1°, $[\alpha]_D^{12·5}$ +3·8 in pyridine. The sugar fraction was dissolved in methanol, filtered, and precipitated from ethanol. 0·1 g was hydrolysed for 4 hr at 90–100° in N-HCl. After neutralization and decolorization with charcoal, the hydrolysate yielded a syrup weighing 10 mg. The sugar, detected with aniline hydrogen phthalate, migrated as a hexose on Oriental No. 50 filter paper irrigated with *n*-butanol–acetic acid–water (4:1:5). The unhydrolysed sugar fraction migrated as α-methylgalactoside under the conditions of HOUGH (1950).

Acidification to congo red with H_2SO_4 of a filtered ethanolic solution containing the free base and cooling overnight, yielded 1·45 gm of the base sulphate. The free base, liberated by treatment with N-NaOH and extracted with ether, gave a positive ninhydrin test and a zero iodine number. The molecular weight by the Rast procedure was 296; calculated for dihydrosphingosine, 301. The base sulphate decomposed on heating above 200° and contained 3·37% N; calculated for $C_{18}H_{39}NO_2 \cdot \frac{1}{2}H_2SO_4$ (Mol. Wt. 350·5) N, 3·99%. The free base (0·16 gm) in pyridine, treated with acetic anhydride, yielded 0·1 gm of triacety derivative. It melted at 102–3°. $[\alpha]_D^{11}$ +22·6 in chloroform. Anal. Calc. for $C_{24}H_{45}NO_5$ (Mol. Wt. 427·6) C, 67·41; H, 10·6; N, 3·28%. Found: C, 67·35; H, 10·45; N, 3·26%.

The tribenzoyl derivative was also prepared by treatment of the free base in pyridine with benzoyl chloride. 25 mg of the tribenzoyl derivative were obtained. Anal. Calc. for $C_{39}H_{51}NO_5$ (Mol. Wt. 613·8) C, 76·31; H, 8·38; N, 2·28%. Found: C, 76·04; H, 8·20; N, 2·25%. The melting point (138–9°) was lower than that of tribenzoyldihydrosphingosine reported by CARTER and NORRIS (1947).

From these results it would appear that the base is dihydrosphingosine, isolated for the first time from human brain although previously found in other tissues (LESUK and ANDERSON, 1941; CARTER and NORRIS, 1947).

SUMMARY

(1) A new cerebroside was isolated by fractionation of the glycolipids extracted with ether from human brain tissue.

(2) Hydroxylignoceric acid, a hexose presumed to be galactose, and dihydrosphingosine were identified as its components.

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THE EXCITATION AND DEPRESSION OF SPINAL NEURONES BY STRUCTURALLY RELATED AMINO ACIDS

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THE anions of aspartic and glutamic acids have potent excitatory action upon spinal neurones (CURTIS, PHILLIS and WATKINS, 1960); this is in marked contrast to the strongly depressant action exhibited by their respective α-decarboxylation products, β-alanine and γ-amino-*n*-butyric acid (GABA) (CURTIS, PHILLIS and WATKINS, 1959). The latter amino acids cause no significant change in the membrane resting potential of motoneurones, which is strong evidence that these substances are not inhibitory transmitter agents within the mammalian spinal cord. On the other hand, the excitant amino acids cause depolarization of spinal neurones, and consequently one of them or a related substance may have excitatory transmitter function within the nervous system. This possibility has led to a survey of the activity of compounds structurally related to both series of amino acids.

METHODS

A detailed account of the general methods employed has appeared previously (CURTIS, PERRIN and WATKINS, 1960; CURTIS, PHILLIS and WATKINS, 1959, 1960.) All experiments were carried out upon motoneurones, Renshaw cells or dorsal horn interneurones within the lumbar region of the spinal cord of the cat lightly anaesthetized with pentobarbital sodium. The amino acids were applied ionophoretically from double-barrelled, five-barrelled, or co-axial micro-electrodes, one barrel of which was employed for recording responses of the neurones. Each substance was tested upon at least three neurones in the one animal; most were re-tested in freshly prepared electrodes at other times upon neurones in another animal. To avoid the uncertainties involved in the use of the five-barrel electrodes (cf. CURTIS *et al.*, 1959) all the substances tested were applied at least once from double-barrel or co-axial electrodes, again upon a minimum of three neurones. Five-barrel electrodes were employed, however, in cases where it was desired to determine the effects of several of the amino acids relative to one another.

The amount of any substance passed ionophoretically from its solution in an electrode is proportional to the current used. To obtain high local concentrations near the tip of the electrode, it was necessary to pass large currents (i.e. greater than 100 mμA) and to maintain such currents for periods of up to 3 min it was necessary, for several reasons, to ensure that the electrode resistances were as low as possible. First, it was observed that the higher the resistance of the electrode (exceeding 100 MΩ), the less the possibility of maintaining constant current flow through it. Often the resistances of such electrodes fluctuated considerably, changes of tenfold being encountered. Thus the usual series resistance of 100 MΩ through which the current was passed to the electrode (CURTIS and ECCLES, 1958) failed to ensure a constancy of this current. Further, the resistance of such electrodes often depended upon the direction of the current flow through them, a finding which was not often observed with electrodes of resistance less than 60 MΩ. The third factor, which technically was of greater importance, was that the passage of current through these high-resistance electrodes generated electrical noise which was effectively recorded by the recording barrel of the electrode since this was of necessity capacitively coupled to the drug containing barrels. It was often difficult in such cases to observe the responses of cells against this background noise.

Low resistance electrodes could be obtained, not only by increasing the tip diameter of the electrodes, but also by increasing the concentration and the degree of ionization of the substance contained therein. Increasing the concentration tends to aid the diffusional escape of material from the tip, but this diffusion is readily overcome by using a backing current, i.e. a current in the reverse direction to that which would pass active ions from the electrode (cf. CASTILLO and KATZ, 1955). In practice a voltage of 0·5 v was applied to the electrode in the appropriate direction. This appeared adequate to prevent leakage of substance from the tip and could be reduced in those cases where the amino acid ion either excited or depressed cells until the effects of the amino acid diffusing from the electrode could be observed. When amino acids appeared to have no action upon spinal cells, either as anions or cations, this backing voltage was removed completely to determine the effect of diffusion alone.

The maximal size of the electrode tip was limited because of the cell damage which might occur in the spinal cord. Usually the individual tip diameters of double barrel electrodes was 3–5 μ and those of five-barrel electrodes 2–3 μ, whereas the outer barrel of a co-axial pair often had a diameter of 10 μ. However, in the last case most of the orifice was occupied by the inner barrel, which projected 50–60 μ beyond the outer barrel when the electrode was being used for the simultaneous intracellular recording of responses due to the extracellular application of the ionized substance. When these electrodes were used to study the action of a substance upon interneurones (see below), outer-barrel tip diameters of 3–4 μ were used, the inner barrel in this case projecting by only 2–4 μ.

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Because of these rather rigid limitations upon the diameter of the tips of the electrodes, and since many of the substances used were zwitterions, it was essential to use a concentrated solution of each amino acid and to adjust the pH of the solutions so that a considerable predominance of either cationic or anionic species of the amino acid was produced. Amino monocarboxylic acids were more effectively applied as cations, the requisite ionic character being obtained by adjustment of their saturated aqueous solution to pH 3–4 with HCl. This conferred a net positive (cationic) charge upon the amino acid to an extent dependent upon the actual pH selected and the acid dissociation constant of the carboxyl group of the amino acid. The pK_a of the carboxylic acid group of α-amino acids is usually between 2 and 2·5, that of β-amino acids is in the region of 3·5, and that of amino acids in which the amino group is separated by three or more carbon atoms from the carboxyl group is about 4 to 4·5 (CONWAY, 1952). Adequate cationic character is obtained at pH 3 for α-amino acids and at pH 3–4 for other amino acids. At a given pH the degree of ionization, and hence the proportion of the cationic current carried by amino acid in relation to that carried by hydrogen ion, is greater for acids having higher pK_a values for the carboxylic group. Care was exercised, as previously, to avoid solutions having a pH less than 2·5 as the passage of hydrogen ions themselves from such solutions results in excitation of spinal neurones (see later).

On occasion, amino monocarboxylic acids were passed as anions from strongly alkaline solutions in which protonation of the amino group of the acids was negligible. When electrodes were filled with solutions of NaOH exceeding pH 13, no effect was observed on passing hydroxyl ions into the neighbourhood of spinal cells. Difficulty was encountered in preliminary experiments when one barrel of either a double or a five-barrel electrode was filled with either glycine, β-alanine, or GABA in solutions of pH exceeding 13. Since the pK_a of the amino group of these acids is 9·60, 10·19, and approximately 10·4, respectively (CONWAY, 1952), it would be reasonable to expect that they would pass as anions from such solutions. However, not only would the pH of these solutions change owing to the absorption of carbon dioxide from the air over the 36–48 hr necessary for complete filling (CURTIS and ECCLES, 1958) but it is possible that interaction between the strongly alkaline solution and the glass wall of the electrode would also contribute to such a pH change of the solution at the electrode tip. Although it was technically impossible to measure the pH of the solution in the extreme tip of the electrode, this pH may in fact have been much lower than the desired high value since the substances still behaved ionophoretically as cations. However, when co-axial electrodes were filled and assembled immediately before use, these substances could readily be passed as anions from solutions of pH 12·5 and above, in which case the same depressant action upon cells was observed as when the substances were passed as cations from solutions at pH 3–4. These observations exclude the possibility that the observed depressant effects on neuronal responses were due to the flow of current rather than to an action of the applied substances. The observation that taurine could be passed readily as an anion from solutions of pH 8 is probably due to the low pK_a (8·8) (CONWAY, 1952) of the amino group of taurine which allows the attainment of a sufficiently high concentration

of the anionic species in solutions of such relatively low pH. The failure to pass glycine, β-alanine, or GABA as anions from solutions having a pH in the range 9–12·5, even in co-axial electrodes, may be related not only to pH changes at the tip of the electrode, but also to the observation that when sodium or chloride ions were passed from electrodes containing approximately equal concentrations of GABA and sodium chloride at pH 6·5, 7·5, and 8·5 (straddling the isoelectric point of GABA), no depression of neuronal activity was observed during passage of anionic currents through the electrode, in contrast to that observed during the passage of currents in the reverse direction. This seemingly cationic behaviour of GABA would be expected from the concomitant movement of water molecules and sodium ions owing both to the hydration of the latter and to electro-osmosis arising from the presence of an electrical double layer at the inner glass surfaces of the electrode tip (JENERICK and GERARD, 1953; CURTIS, PERRIN and WATKINS, 1960)

The second acidic group of amino dicarboxylic acids renders these substances readily obtainable as anions. Routinely, the pH of their aqueous solutions was adjusted to about 8–8·5 with NaOH, (CURTIS, PHILLIS and WATKINS, 1960), although in a few cases KOH was used because the greater solubility of the potassium salts permitted the preparation of more concentrated solutions.

In many cases the choice of pH and thus of the ionic character of the substance being tested was limited by the nature of the compound. The acidic dissociation constants and stability of each compound were always taken into account in selecting a pH which, while conferring an adequate charge on the substance, nevertheless gave maximal protection from possible hydrolysis and from spurious effects due to hydrogen and metal ions. No effects were ever observed when the currents applying an active substance were reversed, i.e. when chloride or alkali metal ions were ionophoretically applied from the electrode instead of the active substance. Use was made of diffusion and electro-osmosis where it was desired to test those substances which do not possess ionizable groups. Observations with electrodes filled with active excitant amino acid ions such as glutamate show that, in the absence of a backing current, sufficient active ion diffuses from the electrode to excite cells near the electrode tip (CURTIS, PHILLIS and WATKINS, 1960). Passage of neutral substances from the tip of an electrode can be facilitated by the concomitant presence of NaCl, since by ionophoretically applying Na^+ into the medium the outward flow of water molecules thus induced increases the rate of diffusion of the unionized substance. Such a phenomenon has been discussed above with respect to the anomalous behaviour of neutral amino acids in NaOH solutions of pH 9–12·5. Neutral substances of the type presently under discussion were therefore prepared in aqueous solutions containing 1–2 M-NaCl. When currents of 200–300 mμA in either direction and prolonged free diffusion of material from the electrode both failed to influence the behaviour of cells located near the electrode tip, it was concluded that these substances had no action upon the cells. The observed results in these cases, although probably valid, are less certain than those in which the substances could be obtained in anionic or cationic form.

Whatever the ionic nature of the amino acids in the electrode, it is reasonable to assume that on reaching the external medium after ionophoretic application, they took up an ionic form determined only by the local pH. Consideration of the respective ionization constants indicates that, when the neutral amino acids were passed from solutions of pH 3, the ion leaving the electrode would be the cation (e.g. GABA: $H_3N^+(CH_2)_3COOH$), whilst when dicarboxylic amino acids were passed from pH 8 solutions, the ion would be predominantly the mono-anion (e.g. glutamate: $^-OOC(CH_2)_2 \cdot CH(NH_3^+)COO^-$). The passage of the amino acids in the cationic form would result, in the neutral external medium, in the release of one proton for each ion passed. It was noticed that when applying such amino acid cations, except in those cases where the amino acid was a strong depressant, cell excitation of a characteristic type invariably occurred. This excitation was similar to that observed for hydrogen ion alone (CURTIS *et al.*, 1959), having a long latency of 10–20 seconds. It was not observed when the amino acid was an active depressant, presumably because the cell responses were already blocked by this more rapid action. These excitant effects provide confirmatory evidence, if needed, that the amino acid cation was leaving the electrode. In the case of the acidic amino acids passed from solutions of pH 8, the ions do not alter significantly on reaching the external solution. The application of a predominance of the di-anionic species (e.g. glutamate: $^-OOC(CH_2)_2 \cdot CH(NH_2)COO^-$) from solutions of pH above 10 would tend to effect a localized increase in the pH of the medium owing to the protonation of the amino group therein. This would be unlikely to affect neurones as hydroxyl ions passed from solutions of pH 13 and greater have no action upon spinal cells (CURTIS, PHILLIS and WATKINS, unpublished observations).

Since the filling of two- or five-barrel electrodes involved a period of at least 48 hr during which the solution of the substance under test diffused downwards towards the electrode tip, co-axial electrodes were used for the ionophoretic application of substance of suspected low-stability in solution. The outer barrels could be filled by suction and the complete electrode assembled immediately prior to use. In all cases, where for any reason breakdown of the substance in the electrode was suspected, paper chromatography was employed to check the electrode contents. Most of the amino acids were obtained commercially or synthesized by known methods. The rest were donated by investigators working in similar fields. The substances synthesized in this laboratory were all analytically pure. Other samples were not checked unless there was reason to suspect contamination; in such cases elemental analysis and paper chromatography served as criteria of purity.

RESULTS

In most cases, the results reported are based upon observations on the negative–positive extracellular spike potentials of interneurones. These gave evidence whether a particular substance excited cells or prevented their synaptic activation; however, little or no indication can be obtained extracellularly of inhibitory synaptic action and intracellular records are then necessary. By means of co-axial electrodes, amino acid ions could be applied through the outer electrode to the external surface of motoneurones, whilst intracellular responses were recorded with the 0·6 M-K_2SO_4-containing inner electrode. Such intracellular electrodes enable post-synaptic potentials, both excitatory and inhibitory, to be recorded undistorted by ions diffusing from the intracellular electrode (COOMBS, ECCLES and FATT, 1955). Co-axial electrodes were used to investigate the action of certain acids, especially the higher ω-amino monocarboxylic acids suggested to have specific effects upon inhibitory synaptic transmission of cortical cells (PURPURA *et al.*, 1959). In particular, ω-amino caprylic acid had no action upon interneurones and was not observed to have any excitant or depressant action upon motoneurones nor any blocking effect upon inhibitory post-synaptic potentials.

The routine use of co-axial electrodes for the determination of the effect of a particular substance upon the motoneuronal membrane was impracticable because of the associated technical difficulties. Consequently the usual test neurone was the interneurone because of the ease with which these cells could be located and extracellular records obtained of the orthodromically evoked spike potentials. The observations of the action of GABA and β-alanine (CURTIS *et al.*, 1959) and of glutamate, aspartate, and cysteate ions (CURTIS, PHILLIS and WATKINS, 1960) upon the extracellularly recorded spike responses of these cells and of Renshaw cells were consistent with the observations of the action of these substances upon the intracellularly recorded responses of motoneurones. This uniformity of action of a particular amino acid upon these different neurones was also observed for β-aminoglutaric acid, γ-amino-β-hydroxybutyric acid, and taurine. It is permissible to assume, therefore, that the effects of other structurally similar amino acids would be the same upon motoneurones and Renshaw cells as those observed upon interneurones.

A direct comparison of the activity of several substances was possible only when they were in different barrels of the same multibarrel electrode. Thus the use of five-barrel electrodes permitted an analysis of the actions of four different amino acids upon any single cell, the central barrel being used for recording. Observations made in this fashion were identical with those made with double-barrel electrodes in which only one substance could be applied to a cell. In most cases, a comparison was based upon observations of extracellularly recorded spike potentials of equal

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magnitude, these being in the range 0·5–1 mv. However, the activity of a particular compound in different experiments was always consistent irrespective of the spike size, the only difference being a longer latency of action where spike sizes were smaller. When these were very small the cell was probably located at a considerable distance from the electrode, and, correspondingly, even strong excitant or depressant substances were ineffective. When spike sizes were larger than 2–3 mv, the size often varied with the flow of current out of the electrode, irrespective of the ion being applied. It was assumed that the electrode was then very close to the cell and indeed, in many of such cases, impalement occurred with subsequent destruction of the cell. When the size of spikes was influenced by current flow in this manner, the electrode was cautiously withdrawn from the neighbourhood of the cell until further testing indicated that the spike size was independent of current flow.

In assigning an index of activity to the various compounds, consideration was given to the magnitude of the current which had to be passed to obtain an effect (an indication of the amount of substance being administered), and the latency, intensity, and duration of the effect, if any. With the excitatory agents, the frequency of firing gave a measure of the activity as also did the rapidity with which an eventual block could be produced (CURTIS, PHILLIS and WATKINS, 1960). Relative depressant activity was estimated from the reduction in the number of spontaneous and synaptically evoked spikes and from the diminution of the field potentials generated by orthodromic or antidromic activation of groups of cells. In the accompanying Tables the actions of the various amino acids have been compared by using ionophoretic currents of 100–150 mμA. The symbol '+' indicates an excitatory action upon neurones, whereas '−' denotes a depressant action, the symbols being enclosed within brackets when the effect was of doubtful significance. Zero (0) indicates the absence of any observable effect, apart from the late excitation which was observed with most neutral amino acids and associated with hydrogen ion concentration (see Methods). Although the comparative rating of the intensity of action by means of a number of symbols is only approximate, there was a clear cut distinction between those acids which strongly excited or depressed interneurones and those which were weak or inert. With the latter substances, larger currents and closer proximity of the electrode to the cell failed to demonstrate any action, apart from the late excitation previously mentioned as due to hydrogen ions.

Apart from two exceptions, the time courses of the excitation or depression of interneurones produced by the active substances (Tables 1 and 2) were similar to those of glutamate and GABA respectively (CURTIS *et al.*, 1959, 1960). Thus the action of most substances ceased within a few hundred msec of the termination of the ionophoretic currents. However, although N-methyl-β-alanine and N-methyl-DL-aspartate had slightly weaker actions than β-alanine and L-aspartate respectively, the actions of these methylated amino acids continued for 10–20 sec after the applying currents were terminated.

Tables 1 and 2 summarize the activities of compounds so selected to elucidate best the structure–activity relationships of the excitatory and depressant amino acids respectively. With very few exceptions modification of the aspartic and glutamic acid molecular structures led to profound impairment or complete abolition of excitatory activity (Table 1). Certain changes, however, did not result in severe attenuation of activity. These comprise: (i) configurational change at the aminated

TABLE 1.—STRUCTURE–ACTIVITY RELATIONSHIPS OF THE EXCITATORY AMINO ACIDS

Feature under investigation	No.	Compound	Structure	pH[1]	Predominant ion species	Action[2]	Reference compounds[3]
A. Distance between the amino group and the most distal (ω) carboxyl group	(1)	Aminomalonic acid	$HOOC{\cdot}CH(NH_2){\cdot}COOH$	8(NaOH)	anion	+	
	(2)	DL-Aspartic acid	$HOOC{\cdot}CH_2{\cdot}CH(NH_2){\cdot}COOH$	8(NaOH)	anion	+++	
	(3)	DL-Glutamic acid	$HOOC{\cdot}(CH_2)_2{\cdot}CH(NH_2){\cdot}COOH$	8,13(NaOH)	anion	+++	
	(4)	DL-α-Aminoadipic acid	$HOOC{\cdot}(CH_2)_3CH(NH_2){\cdot}COOH$	8(NaOH)	anion	+	
	(5)	DL-α-Aminopimelic acid	$HOOC{\cdot}(CH_2)_4{\cdot}CH(NH_2){\cdot}COOH$	8(NaOH)	anion	+	
B. Configuration at aminated carbon atom	(6)	D-Aspartic acid	See (A)	8(NaOH)	anion	++(+)	DL-Aspartic acid (2)
	(7)	L-Aspartic acid		8(NaOH)	anion	+++	
	(8)	D-Glutamic acid		8(NaOH)	anion	++(+)	DL-Glutamic acid (3)
	(9)	L-Glutamic acid		8(NaOH)	anion	+++	
C. Variation of the ω-acidic group	(10)	L-Cysteic acid	$HO_3S{\cdot}CH_2{\cdot}CH(NH_2){\cdot}COOH$	8(NaOH)	anion	+++	L-Aspartic acid (7)
	(11)	L-Cysteine sulphinic acid	$HO_2S{\cdot}CH_2{\cdot}CH(NH_2){\cdot}COOH$	8(NaOH)	anion	+++	L-Aspartic acid (7)
	(12)	O-Phosphoryl-L-serine	$H_2O_3PO{\cdot}CH_2{\cdot}CH(NH_2){\cdot}COOH$	8(NaOH)	anion	0	L-Glutamic acid (9), L-Aspartic acid (7)
	(13)	DL-*o*-Tyrosine	$\cdot CH_2{\cdot}CH(NH_2){\cdot}COOH$	12(NaOH)	anion	0	DL-Glutamic acid (3)
D. Relative disposition of amino and carboxyl groups	(14)	β-Aminoglutaric acid	$HOOC{\cdot}CH_2{\cdot}CH(NH_2){\cdot}CH_2{\cdot}COOH$	8(NaOH)	anion	++(+)	Glutamic acid (3, 8, 9)
	(15)	DL-β-Aminoadipic acid	$HOOC{\cdot}(CH_2)_2{\cdot}CH(NH_2){\cdot}CH_2{\cdot}COOH$	8(NaOH)	anion	0	DL-α-Aminoadipic acid (4)
	(16)	γ-Aminopimelic acid	$HOOC{\cdot}(CH_2)_2{\cdot}CH(NH_2){\cdot}(CH_2)_2{\cdot}COOH$	8(NaOH)	anion	0	DL-α-Aminopimelic acid (5)
	(17)	2-Aminoethylmalonic acid	$HOOC{\cdot}CH(COOH){\cdot}CH_2CH_2{\cdot}NH_2$	8(NaOH)	anion	0	Glutamic acid (3, 8, 9)
	(18)	DL-2-(Aminomethyl)succinic acid	$HOOC{\cdot}CH_2{\cdot}CH(COOH){\cdot}CH_2{\cdot}NH_2$	8(NaOH)	anion	+	DL-Glutamic acid (3)
	(19)	Iminodiacetic acid	$HOOC{\cdot}CH_2{\cdot}NH{\cdot}CH_2COOH$	8(NaOH)	anion	0	Aspartic acid (2, 6, 7)
E. Substitution in the carbon chain	(20)	α-Methyl-DL-glutamic acid	$HOOC{\cdot}(CH_2)_2{\cdot}C(CH_3)(NH_2){\cdot}COOH$	8(NaOH)	anion	0	DL-Glutamic acid (3)
	(21)	β-Hydroxyglutamic acid	$HOOC{\cdot}CH_2{\cdot}CH(OH){\cdot}CH(NH_2){\cdot}COOH$	8(NaOH)	anion	++	DL-Glutamic acid (3)
	(22)	*meso*-α:β-Diaminosuccinic acid	$HOOC{\cdot}CH(NH_2){\cdot}CH(NH_2){\cdot}COOH$	11,13(KOH)[4]	anion	0	Aspartic acid (2, 6, 7)
	(23)	L-α-ε-Diaminopimelic acid	$HOOC{\cdot}CH(NH_2){\cdot}(CH_2)_3{\cdot}CH(NH_2){\cdot}COOH$	11,13(KOH)[4]	anion	0	DL-α-Aminopimelic acid (5)
F. N-Alkylation	(24)	N-Methyl-DL-aspartic acid	$HOOC{\cdot}CH_2{\cdot}CH(NHCH_3){\cdot}COOH$	8(NaOH)	anion	++[5]	DL-Aspartic acid (2)
	(25)	N:N-Dimethyl-DL-aspartic acid	$HOOC{\cdot}CH_2{\cdot}CH{\cdot}[N(CH_3)_2]COOH$	8(NaOH)	anion	+	N-Methyl-DL-aspartic acid (24)
	(26)	N-Methyl-DL-glutamic acid	$HOOC{\cdot}(CH_2)_2{\cdot}CH(NHCH_3){\cdot}COOH$	8(NaOH)	anion	0	DL-Glutamic acid (3)
	(27)	N:N-Dimethyl-L-glutamic acid	$HOOC{\cdot}(CH_2)_2CH[N(CH_3)_2]COOH$	8(NaOH)	anion	+[6]	N-Methyl-DL-glutamic acid (26)
	(28)	α-Glutarobetaine	$HOOC{\cdot}(CH_2)_2CH[N^+(CH_3)_3]COO^-$	8(NaOH)	anion	0	N:N-Dimethyl-L-glutamic acid (27)

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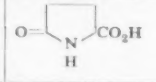
G. Replacement of the amino group by the formamidino group	(29)	N-Formimino-L-aspartic acid	$HOOC{\cdot}CH_2{\cdot}CH(NHCH{=}NH){\cdot}COOH$	8(NaOH)	anion	++	L-Aspartic acid (7)
H. N-Acylation	(30)	N-Acetyl-L-aspartic acid	$HOOC{\cdot}CH_2{\cdot}CH(NHCOCH_3){\cdot}COOH$	7(NaOH)	anion	0	L-Aspartic acid (7)
	(31)	N-Carbamoyl-DL-aspartic acid	$HOOC{\cdot}CH_2{\cdot}CH(NHCONH_2){\cdot}COOH$	7(NaOH)	anion	0	DL-Aspartic acid (2)
	(32)	N-Acetyl-L-glutamic acid	$HOOC{\cdot}(CH_2)_2{\cdot}CH(NHCOCH_3){\cdot}COOH$	7(NaOH)	anion	0	L-Glutamic acid (9)
	(33)	N-Carbamoyl-L-glutamic acid	$HOOC{\cdot}(CH_2)_2{\cdot}CH(NHCONH_2){\cdot}COOH$	7(NaOH)	anion	0	L-Glutamic acid (9)
I. ω-Amide, ester and hydrazide linkages	(34)	L-Asparagine	$HOOC{\cdot}CH(NH_2){\cdot}CH_2CONH_2$	3(HCl)	cation	0	L-Aspartic acid (7)
	(35)	L-Glutamic acid-γ-methyl ester	$HOOC{\cdot}CH(NH_2){\cdot}(CH_2)_2{\cdot}COOCH_3$	3(HCl)	cation	0	L-Glutamic acid (9)
	(36)	L-Glutamine	$HOOC{\cdot}CH(NH_2){\cdot}(CH_2)_2{\cdot}CONH_2$	3(HCl)	cation	0	L-Glutamic acid (9)
	(37)	L-Glutamic acid-γ-methylamide	$HOOC{\cdot}CH(NH_2){\cdot}(CH_2)_2{\cdot}CONHCH_3$	3(HCl)	cation	0	L-Glutamic acid (9)
	(38)	L-Glutamic acid-γ-dimethylamide	$HOOC{\cdot}CH(NH_2){\cdot}(CH_2)_2{\cdot}CON(CH_3)_2$	3(HCl)	cation	0	L-Glutamic acid (9)
	(39)	γ-L-Glutamylglycine	$HOOC{\cdot}CH(NH_2){\cdot}(CH_2)_2{\cdot}CONH{\cdot}CH_2{\cdot}COOH$	8(NaOH)	anion	0	L-Glutamic acid (9)
	(40)	L-Glutamic acid-γ-hydrazide	$HOOC{\cdot}CH(NH_2){\cdot}(CH_2)_2{\cdot}CONHNH_2$	3(HCl)	cation	0	L-Glutamic acid (9)
J. α-Amides and ester linkages	(41)	L-*Iso*glutamine	$HOOC{\cdot}(CH_2)_2{\cdot}CH(NH_2){\cdot}CONH_2$	3(HCl)	cation	0	L-Glutamic acid (9)
	(42)	L-Glutamic acid-α-methyl ester	$HOOC{\cdot}(CH_2)_2{\cdot}CH(NH_2){\cdot}COOCH_3$	3(HCl)	cation	0	L-Glutamic acid (9)
	(43)	α-L-Glutamylglycine	$HOOC(CH_2)_2{\cdot}CH(NH_2){\cdot}CONH{\cdot}CH_2{\cdot}COOH$	8(NaOH)	anion	0	L-Glutamic acid (9)
K. Cyclization	(44)	Pyroglutamic acid		8(NaOH)	anion	0	Glutamic acid (3, 8, 9)
L. Absence of the amino group	(45)	Malonic acid	$HOOC{\cdot}CH_2{\cdot}COOH$	8(NaOH)	anion	0	Aminomalonic acid (1)
	(46)	Succinic acid	$HOOC{\cdot}(CH_2)_2{\cdot}COOH$	8(NaOH)	anion	0	Aspartic acid (2, 6, 7)
	(47)	Glutaric acid	$HOOC{\cdot}(CH_2)_3COOH$	8(NaOH)	anion	0	Glutamic acid (3, 8, 9)
	(48)	L-Malic acid	$HOOC{\cdot}CH_2{\cdot}CH(OH){\cdot}COOH$	8(NaOH)	anion	0	Aspartic acid (2, 6, 7)
M. Absence of both carboxyl groups	(49)	Ethylamine	$CH_3{\cdot}CH_2{\cdot}NH_2$	4(HCl)	cation	0	Aspartic acid (2, 6, 7)
	(50)	Propylamine	$CH_3(CH_2)_2\ NH_2$	4(HCl)	cation	0	Glutamic acid (3, 8, 9)
N. Absence of one carboxyl group		α or ω Monoamino monocarboxylic acids—See depressant amino acids (Table 2)					

[1] The pH of the saturated aqueous solution after adjustment with the acid or alkali shown in parentheses.
[2] The effect on interneurones. See text for meaning of symbols.
[3] The number of each reference compound is given in parentheses after the compound.
[4] The potassium salts are more soluble than the sodium salts.
[5] Longer acting than the reference compound, i.e. longer duration of continued action after cessation of application.
[6] This substance contained traces of the unmethylated and monomethylated compounds which could not be completely removed by recrystallization.

carbon atom of aspartic acid (Table 1: 2, 6 and 7) and glutamic acid (Table 1: 3, 8 and 9); (ii) the replacement of the β-carboxyl group of L-aspartic acid by the sulphonic acid group, as in L-cysteic acid (Table 1: 10) or by the sulphinic acid group, as in L-cysteine sulphinic acid (Table 1: 11); (iii) the transfer of the amino group from the α-position of glutamic acid to the β-position (β-aminoglutaric acid, Table 1: 14); (iv) the introduction of an hydroxyl group into the β-position of the glutamic acid molecule (β-hydroxyglutamic acid, Table 1: 21), or of an N-methyl or N-formimino group into the aspartic acid structure (N-methyl-DL-aspartic acid, Table 1: 24 and N-formimino-L-aspartic acid, Table 1: 29 respectively).

The structure–activity relationships demonstrated by the results listed in Table 1 may be summarized according to the specific sections of the Table as follows:

Section A. Variation of the number of carbon atoms in the chain connecting the α-amino and ω-carboxyl groups shows that peak activity is associated with a separation of these groups by two or three carbon atoms (glutamic and aspartic acids, numbers 2 and 3 respectively in Table 1).

Section B. There is little demonstrable difference in the activities of the aspartic and glutamic acid enantiomorphs, but the L-isomer appears to be slightly more effective in both cases.

Section C. The sulphonic and sulphinic acid groups can each adequately replace the β-carboxyl group of L-aspartic acid (Table 1: 7); L-cysteic acid (Table 1: 10) and L-cysteine sulphinic acid (Table 1: 11) both being strongly excitatory. On the other hand neither the strongly acidic phosphoryl group of O-phosphoryl-L-serine (Table 1: 12) nor the weakly acidic phenolic hydroxyl group of DL-*o*-tyrosine (Table 1: 13) can replace the γ-carboxyl group of glutamic acid.

Section D. Retaining the optimum separation of two or three carbon atoms of one carboxyl group from the amino group and varying the position of the other carboxyl group leads mostly to inactive compounds. The sole exceptions are the strongly excitatory β-aminoglutaric acid (Table 1: 14) and the less active 2-(aminomethyl)-succinic acid (Table 1: 18).

Section E. The introduction of small substituents into the carbon chain linking the two carboxyl groups reduces the activity. This effect is much more marked with an α-methyl than with a β-hydroxyl substituent in the glutamic acid molecule. (Compare α-methyl-DL-glutamic acid, Table 1: 20 and β-hydroxyglutamic acid, Table 1: 21). A second amino group, in the α'-position, abolishes the activity of both aspartic and α-aminopimelic acids (compounds 22 and 23 respectively in Table 1).

Section F. Alkylation of the amino group also results in lowered activity but the effect is minimal in the case of a single methyl group. The longer duration of action of N-methyl-DL-aspartic acid (Table 1: 24) compared with that of aspartic acid is of particular interest.

Section G. N-formimino-L-aspartic acid (Table 1: 29) is only slightly less active than aspartic acid and about equal in activity to N-methyl-DL-aspartic acid (Table 1: 24).

Sections H, I, J, K. Both acylation of the amino group, and involvement of the α and/or ω-carboxyl terminals in ester or amide linkages result in complete loss of activity.

Sections L, M, N. Compounds which lack the amino group or both carboxyl groups are all inactive. Loss of just one of the carboxyl groups, in particular that from the α-position, leads to the series of depressant amino acids (Table 2).

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The structure–activity relationships of the depressant neutral amino acids show a very close correlation with those of the excitatory acidic amino acids, as can be seen from the following summary of the results listed in the various sections of Table 2.

Section A. For depressant action the optimal separation of the carboxyl and amino groups of the ω-amino monocarboxylic acids is two or three carbon atoms, the same as that between the ω-carboxyl group and the amino group of the excitatory amino dicarboxylic acids (cf. Table 1: Section A). No activity of ω-aminocaprylic acid could be demonstrated.

Section B. The size and number of substituents on the carbon atom bearing the amino group profoundly influences the depressant action. Very little, if any, of this depressant activity is retained when this substitution is more extensive than a single methyl group (compare DL-α-alanine; Table 2: 7, with the other compounds of this group). Only one corresponding compound was available in the acidic amino acid series, this being α-methyl-DL-glutamic acid (Table 1: 20) which likewise showed markedly reduced excitatory activity in comparison with the reference compound, DL-glutamic acid.

Section C. Substitution upon intermediate carbon atoms is also detrimental to depressant action though perhaps less so than substitution upon the aminated carbon atom.

Section D. There seems to be little difference in activity between the D- and L-enantiomorphs of α-alanine (Table 2: 24 and 25 respectively). This accords with the previously mentioned observation that D- and L-enantiomorphs of both glutamic and aspartic acid also show approximately the same activity. (Table 1, Section B).

Section E. The reduction of depressant activity upon increasing N-alkylation of the amino dicarboxylic acids (cf. Table 1, Section F) is also followed closely among the N-alkylated amino monocarboxylic acids. In view of its reported acetylcholine-like properties (HOSEIN and MCLENNAN, 1959), γ-butyrobetaine (Table 2: 33) was also tested on Renshaw cells where it was not shown to have any excitatory activity.

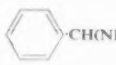
Sections F, G. Acylation of the amino group abolishes depressant activity as was the case with the excitatory amino acids (cf. Table 1, Section H). Involvement of the carboxyl group in ester or amide linkages likewise results in inactive compounds in both series (cf. Table 1, Sections I, J).

Section H. The sulphonic acid group is again able to replace the carboxyl group (compare Table 2: 49 with Table 1, Section C). It is interesting, however, that N-methylation of taurine (N-methyltaurine, Table 2: 50) abolishes the depressant activity whilst that of β-alanine (N-methyl-β-alanine, Table 2: 28) merely reduces it.

Section I. Replacement of the amino group of glycine by the formamidino group (N-formiminoglycine Table 2: 54) reduces the depressant potency while replacement by a guanidino group (glycocyamine, Table 2: 55) results in greater loss of activity. Creatine (Table 2: 56) and γ-guanidinobutyric acid (Table 2: 57) were inactive by this method of testing (cf. PURPURA *et al.*, 1959; EDWARDS and KUFFLER, 1959). Amongst the amino dicarboxylic acids, the N-formimino group was likewise seen to reduce, but not to abolish, excitatory activity (Table 1, Section G). No guanidino dicarboxylic acids were available.

Section J. Internal amide formation involving the terminal amino and carboxyl groups leads to cyclic compounds which are probably inactive, although two of the examples of this group are neutral and thus had necessarily to be applied by diffusion

TABLE 2.—STRUCTURE–ACTIVITY RELATIONSHIPS OF THE DEPRESSANT AMINO ACIDS

Feature under investigation	No.	Compound	Structure	pH[1]	Predominant ion species	Action[2]	Reference compounds[3]
A. Distance between the carboxyl group and the amino group	(1)	Glycine	$H_2N{\cdot}CH_2{\cdot}COOH$	3(HCl)	cation	– –	
	(2)	β-Alanine	$H_2N{\cdot}(CH_2)_2{\cdot}COOH$	3(HCl)	cation	– – –	
	(3)	γ-Amino-*n*-butyric acid	$H_2N{\cdot}(CH_2)_3{\cdot}COOH$	3(HCl)	cation	– – –	
	(4)	δ-Amino-*n*-valeric acid	$H_2N{\cdot}(CH_2)_4{\cdot}COOH$	3(HCl)	cation	– –	
	(5)	ε-Aminocaproic acid	$H_2N{\cdot}(CH_2)_5{\cdot}COOH$	4(HCl)	cation	–	
	(6)	ω-Aminocaprylic acid	$H_2N{\cdot}(CH_2)_7{\cdot}COOH$	4(HCl)	cation	0	
B. Alkyl substitution upon the aminated carbon atom	(7)	DL-α-Alanine	$CH_3{\cdot}CH(NH_2){\cdot}COOH$	3(HCl)	cation	– –	Glycine (1)
	(8)	DL-α-Amino-*n*-butyric acid	$CH_3{\cdot}CH_2{\cdot}CH(NH_2){\cdot}COOH$	3(HCl)	cation	0	Glycine (1)
	(9)	DL-β-Amino-*n*-butyric acid	$CH_3{\cdot}CH(NH_2){\cdot}CH_2{\cdot}COOH$	3(HCl)	cation	–	β-Alanine (2)
	(10)	DL-α-Amino*iso*butyric acid	$CH_3{\cdot}C(NH_2)(CH_3){\cdot}COOH$	3(HCl)	cation	0	Glycine (1)
	(11)	DL-, D-and L-Serine	$HO{\cdot}CH_2{\cdot}CH(NH_2){\cdot}COOH$	9(NaOH)	anion	0	Glycine (1)
	(12)	O-Acetyl-DL-serine	$CH_3COO{\cdot}CH_2{\cdot}CH(NH_2){\cdot}COOH$	3(HCl)	cation	0	Glycine (1)
	(13)	L-Cysteine (hydrochloride)	$HS{\cdot}CH_2CH(NH_2){\cdot}COOH(HCl)$	12(NaOH)	anion	0	Glycine (1)
	(14)	DL-Norvaline	$CH_3{\cdot}(CH_2)_2{\cdot}CH(NH_2){\cdot}COOH$	3,12(HCl, NaOH)	cation, anion	0	Glycine (1)
	(15)	L-Valine	$CH_3{\cdot}CH(CH_3){\cdot}CH(NH_2){\cdot}COOH$	12(NaOH)	anion	0	Glycine (1)
	(16)	DL-Norleucine	$CH_3{\cdot}(CH_2)_3{\cdot}CH(NH_2){\cdot}COOH$	12(NaOH)	anion	0	Glycine (1)
	(17)	L-Leucine	$CH_3{\cdot}CH(CH_3){\cdot}CH_2{\cdot}CH(NH_2){\cdot}COOH$	3,12(HCl, NaOH)	cation, anion	0	Glycine (1)
	(18)	DL-*Iso*leucine	$CH_3{\cdot}CH_2{\cdot}CH(CH_3){\cdot}CH(NH_2){\cdot}COOH$	13(NaOH)	anion	0	Glycine (1)
	(19)	β-Phenyl-β-alanine		11(NaOH)	anion	0	β-Alanine (2)
C. Substitution upon intermediate carbon atoms	(20)	γ-Amino-β-hydroxybutyric acid	$H_2N{\cdot}CH_2{\cdot}CH(OH){\cdot}CH_2{\cdot}COOH$	3(HCl)	cation	– –	γ-Amino-*n*-butryic acid (3)
	(21)	DL-β-Amino*iso*butyric acid	$H_2N{\cdot}CH_2{\cdot}CH(CH_3){\cdot}COOH$	3(HCl)	cation	– –	β-Alanine (2)
	(22)	DL-2:2-Dimethyl-β-alanine	$H_2N{\cdot}CH_2C(CH_3)_2COOH$	3(HCl)	cation	0	β-Alanine (2)
	(23)	2:4-Diaminobutyric acid	$H_2N{\cdot}(CH_2)_2{\cdot}CH(NH_2){\cdot}COOH$	3(HCl)	cation	–	γ-Amino-*n*-butyric acid (3), DL-α-Amino-*n*-butyric acid (8)

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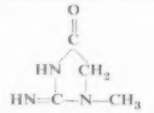
D. Configuration at the aminated carbon atom	(24)	D(−)-α-Alanine	See B above	3(HCl)	cation	− −	DL-α-Alanine (7)
	(25)	L(+)-α-Alanine		3(HCl)	cation	− −	DL-α-Alanine (7)
E. N-Alkylation	(26)	N-Methylglycine (sarcosine)	$CH_3{\cdot}NH{\cdot}CH_2{\cdot}COOH$	3(HCl)	cation	−	Glycine (1)
	(27)	N:N-Dimethylglycine	$(CH_3)_2N{\cdot}CH_2{\cdot}COOH$	4(HCl)	cation	0	N-Methylglycine (26)
	(28)	N-Methyl-β-alanine	$CH_3{\cdot}NH{\cdot}(CH_2)_2{\cdot}COOH$	3(HCl)	cation	− −[4]	β-Alanine (2)
	(29)	N:N-Dimethyl-β-alanine	$(CH_3)_2N{\cdot}(CH_2)_2{\cdot}COOH$	3(HCl)	cation	0	N-Methyl-β-alanine (28)
	(30)	β-Propiobetaine hydrochloride	$(CH_3)_3N^+(CH_2)_2{\cdot}COOH(Cl^-)$	3	cation	0	N:N-Dimethyl-β-alanine (29)
	(31)	N-Methyl-γ-amino-*n*-butyric acid	$CH_3{\cdot}NH{\cdot}(CH_2)_3{\cdot}COOH$	3(HCl)	cation	0	γ-Amino-*n*-butyric acid (3)
	(32)	N:N-Dimethyl-γ-amino-*n*-butyric acid	$(CH_3)_2{\cdot}N{\cdot}(CH_2)_3{\cdot}COOH$	3(HCl)	cation	0	N-Methyl-γ-amino-*n*-butyric acid (31)
	(33)	γ-Butyrobetaine	$(CH_3)_3N^+{\cdot}(CH_2)_3{\cdot}COO^-$	3(HCl)	cation	0	N:N-Dimethyl-γ-aminobutyric acid (32)
F. N-Acylation	(34)	N-Formylglycine	$HCONH{\cdot}CH_2{\cdot}COOH$	8(NaOH)	anion	0	Glycine (1)
	(35)	N-Acetylglycine	$CH_3{\cdot}CONH{\cdot}CH_2{\cdot}COOH$	7(KOH)	anion	0	Glycine (1)
	(36)	Glycylcycine	$H_2N{\cdot}CH_2{\cdot}CONH{\cdot}CH_2{\cdot}COOH$	3(HCl)	cation	0	Glycine (1)
	(37)	α-L-Glutamylglycine	$HOOC{\cdot}(CH_2)_2{\cdot}CH(NH_2){\cdot}CONH{\cdot}CH_2{\cdot}COOH$	8(NaOH)	anion	0	Glycine (1)
	(38)	N-Acetyl-β-Alanine	$CH_3{\cdot}CONH{\cdot}(CH_2)_2{\cdot}COOH$	7(NaOH)	anion	0	β-Alanine (2)
	(39)	N-Acetyl-γ-amino-*n*-butyric acid	$CH_3{\cdot}CONH{\cdot}(CH_2)_3{\cdot}COOH$	7(NaOH)	anion	0	γ-Amino-*n*-butyric acid (3)
	(40)	N-Acetyl-N-methyl-β-alanine	$CH_3{\cdot}CON(CH_3){\cdot}(CH_2)_2{\cdot}COOH$	7(NaOH)	anion	0	N-Methyl β-alanine (28)
	(41)	N-Carbamoyl-β-alanine	$H_2N{\cdot}CONH{\cdot}(CH_2)_2{\cdot}COOH$	6·5(NaOH)	anion	0	β-Alanine (2)
	(42)	β-Alanyl-β-alanine	$H_2N{\cdot}(CH_2)_2{\cdot}CONH{\cdot}(CH_2)_2{\cdot}COOH$	3(HCl)	cation	0	β-Alanine (2)
	(43)	γ-L-Glutamyl-β-alanine	$HOOC{\cdot}CH(NH_2){\cdot}(CH_2)_2{\cdot}CONH{\cdot}(CH_2)_2COOH$	8(NaOH)	anion	0	β-Alanine (2)
G. Modifications of the carboxylic function	(44)	Glycine ethyl ester	$H_2N{\cdot}CH_2{\cdot}COOC_2H_5$	3(HCl)	cation	0	Glycine (1)
	(cf 42)	β-Alanyl-β-alanine	$H_2N{\cdot}(CH_2)_2{\cdot}CONH(CH_2)_2{\cdot}COOH$	3(HCl)	cation	0	β-Alanine (2)
	(45)	Carnosine	$\cdot CH_2{\cdot}CH(COOH){\cdot}NHCO{\cdot}(CH_2)_2NH_2$	4(HCl)	cation	0	β-Alanine (2)
	(46)	γ-Amino-*n*-butyric acid methyl ester (hydrochloride)	$H_2N(CH_2)_3{\cdot}COOCH_3(HCl)$	4(NaOH)	cation	0	γ-Amino-*n*-butyric acid (3)
	(47)	γ-Aminobutyrylcholine (chloride, hydrochloride)	$H_2N{\cdot}(CH_2)_3{\cdot}CO{\cdot}O(CH_2)_2N^+(CH_3)_3(Cl^-)(HCl)$	3	cation	0[5]	γ-Amino-*n*-butyric acid (3)
	(48)	γ-Aminobutyramide (hydrochloride)	$H_2N{\cdot}(CH_2)_3{\cdot}CONH_2(HCl)$	3(HCl)	cation	0	γ-Amino-*n*-butyric acid (3)

TABLE 2 (*contd.*)

Feature under investigation	No.	Compound	Structure	pH[1]	Predominant ion species	Action[2]	Reference compounds[3]
H. Replacement of the carboxyl group by other acidic groups	(49)	Taurine	$H_2N{\cdot}(CH_2)_2{\cdot}SO_3H$	8.12(NaOH)	anion	— —	β-Alanine (2)
	(50)	N-Methyltaurine	$CH_3{\cdot}NH{\cdot}(CH_2)_2SO_3H$	11(NaOH)	anion	0	N-Methyl-β-alanine (28)
	(51)	N-Carbamoyltaurine	$H_2N{\cdot}CONH{\cdot}(CH_2)_2{\cdot}SO_3H$	8(NaOH)	anion	0	N-Carbamoyl-β-alanine (41)
	(52)	Isethionic acid	$HO{\cdot}(CH_2)_2SO_3H$	8(NaOH)	anion	0	
	(53)	O-Phosphorylethanolamine	$H_2N{\cdot}(CH_2)_2{\cdot}OPO(OH)_2$	8(NaOH)	anion	0	β-Alanine (2), γ-Amino-*n*-butyric acid (3)
I. Replacement of the amino group by the formamidino or guanidino group	(54)	N-Formiminoglycine	$HN{=}CH{\cdot}NH{\cdot}CH_2{\cdot}COOH$	3(HCl)	cation	—	Glycine (1)
	(55)	Glycocyamine	$H_2N{\cdot}C(=NH){\cdot}NH{\cdot}CH_2{\cdot}COOH$	3(HCl)	cation	0	Glycine (1)
	(56)	Creatine	$H_2N{\cdot}C(=NH){\cdot}N(CH_3){\cdot}CH_2{\cdot}COOH$	3(HCl)	cation	0	N-Methylglycine (26)
	(57)	γ-Guanidinobutyric acid	$H_2N{\cdot}C(=NH){\cdot}NH(CH_2)_3{\cdot}COOH$	4(HCl)	cation	0	γ-Amino-*n*-butyric acid (3)
J. Cyclization	(58)	Creatinine		4(HCl)	cation	0	Creatine (56)
	(59)	2-Pyrrolidone		6	cation	0[6]	γ-Amino-*n*-butyric acid (3)
	(60)	ϵ-Caprolactam		6	cation	0[6]	ϵ-Aminocaproic acid (5)
K. Absence of the amino group	(61)	Acetic acid	$CH_3{\cdot}COOH$	7(NaOH)	anion	0	Glycine (1)
	(62)	Propionic acid	$CH_3{\cdot}CH_2{\cdot}COOH$	7(NaOH)	anion	0	β-Alanine (2)
L. Absence of the carboxyl group	(63)	Methylamine	$CH_3{\cdot}NH_2$	4(HCl)	cation	0	Glycine (1)
	(64)	Ethylamine	$CH_3{\cdot}CH_2{\cdot}NH_2$	4(HCl)	cation	0	β-Alanine (2)
	(65)	*n*-Propylamine	$CH_3{\cdot}(CH_2)_2{\cdot}NH_2$	4(HCl)	cation	0	γ-Amino-*n*-butyric acid (3)
M. Introduction of a second acidic group		Acidic amino acids	See excitatory amino acids Table 1				

[1] [2] [3] See footnote 1, 2, 3 of Table 1. [4] See footnote (5) Table 1. [5] This substance tested upon interneurones only.
[6] Results with these neutral compounds must be interpreted with caution as the amounts applied by diffusion and electro-osmosis may well have been too low (see Methods).

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and electro-osmosis which introduces uncertainties with respect to the concentrations of the test substance obtained (see Methods). The one analogue within the amino dicarboxylic acid series is obtainable in ionic form, but this compound also displayed no activity (Table 1, Section K).

Sections K, L. Compounds which lack either the amino group or the carboxyl group are inactive (cf. Table 1, Sections L, M).

It has been reported previously that the excitation of interneurones by the acidic amino acid ions is effectively prevented by application of the neutral amino acids (CURTIS, PHILLIS and WATKINS 1960). Table 3 summarizes these results, A indicating

TABLE 3.—ANTAGONISM BETWEEN ACTIVE AMINO ACIDS*

Excitant	Depressant			
	Glycine	β-Alanine	GABA	Taurine
Aspartic acid	A	A	A	A
Glutamic acid	A	A	A	A
Cysteic acid	N	A	A	A
β-Hydroxyglutamic acid	N	A	A	N

* The various combinations were tested upon interneurones.
A = antagonism: N = not tested.

antagonism between the two acids, and N that the combination was not tested. In these experiments it was invariably noticed that simultaneous application of a relatively low concentration of a strong depressant (i. e. applied with a relatively small ionophoretic current) was sufficient to block the excitation of a neurone evoked by a relatively high concentration of a strong excitant. That such antagonism was not due to an interaction between ionophoretic currents of opposite direction was excluded by applying some pairs of antagonists—for example, taurine and glutamic acid, with currents of the same direction from solutions of appropriate pH.

DISCUSSION

The application of a series of related compounds to a particular pharmacological test situation allows an analysis to be made of the structural features of these compounds which are important in producing the observed action. It is essential, however, that such a series of substances be tested at similar concentrations. In this regard it is reasonable to assume that when amino acids were applied ionophoretically into the region of single interneurones from electrodes using identical currents, the same range of extracellular concentrations was obtained. In the present experiments it is evident that since for any one substance consistent activity or inactivity was demonstrated on a series of cells, presumably over a range of not greatly differing concentrations, the results indicate differences in the degree of pharmacological action rather than differences in the attained concentrations.

It has thus been shown that a restricted series of amino acid ions excite spinal

neurones by the production of a membrane depolarization (cf. CURTIS, PHILLIS and WATKINS 1960) whereas a related series of amino acids depresses the cells without altering their membrane potential (CURTIS *et al.*, 1959). The two series can be compared by means of their actions upon spinal interneurones (Tables 1 and 2). A remarkable feature of the structure–activity relationships outlined in these Tables is the reversal, not mere reduction or abolition, of activity when the α-carboxyl group of the excitatory acidic amino acids is lacking. Equally significant is the striking correlation between the actual potency of the excitatory activity of any active acidic amino acid and that of the depressant activity of its α-decarboxylation product (Table 4). Indeed, the relative activity of a particular excitant may usually be accurately predicted from a knowledge of the relative depressant activity of its

TABLE 4.—COMPARISON OF RELATED EXCITATORY AND DEPRESSANT AMINO ACIDS

Acidic amino acid	Table 1 section	Action	α-Decarboxylation product	Table 2 section	Action
Aspartic acid	A	+ + +	β-Alanine	A	− − −
Glutamic acid	A	+ + +	GABA	A	− − −
Cysteic acid	C	+ + +	Taurine	H	− − −
β-Hydroxyglutamic acid	E	+ +	γ-Amino-β-hydroxy-*n*-butyric acid	C	− −
N-Methylaspartic acid	F	+ +	N-Methyl-β-alanine	E	− −
Aminomalonic acid	A	+	Glycine	A	− −
α-Aminoadipic acid	A	+	δ-Aminovaleric acid	A	− −
α-Aminopimelic acid	A	+	ε-Aminocaproic acid	A	−
N:N-Dimethylaspartic acid	F	+	N:N-Dimethyl-β-alanine	E	0
N-Methylglutamic acid	F	0	N-Methyl-γ-amino-*n*-butyric acid	E	0

α-decarboxylation product, and vice versa. Thus glutamic, aspartic, and cysteic acids, the three most active of the excitatory agents, lead respectively to GABA, β-alanine, and taurine, the most depressant amino acids, while the less active excitatory compounds, N-methylaspartic acid and β-hydroxyglutamic acid correspond to the less active depressant agents, N-methyl-β-alanine and γ-amino-β-hydroxybutyric acid. Other examples are given in Table 4.

Consideration of these points leads to the conclusion that the two effects are closely interconnected. An explanation must be provided, however, for the opposite nature of these effects and this must take into account the following features of the actions of the two series of amino acids:

(i) Only very limited changes in the structure of the amino acids permit retention of their activity. A particular structural modification to corresponding compounds within the excitant and depressant series has a remarkably similar effect in each series with respect to the activities of the modified, compared with the unmodified, compounds.

(ii) The excitatory action of the acidic amino acids is the direct result of a membrane depolarization (CURTIS, PHILLIS and WATKINS, 1960).

(iii) The depressant amino acids produce no change in membrane potential and

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diminish both excitatory postsynaptic potentials (EPSP) and inhibitory postsynaptic potentials (IPSP) (CURTIS *et al.*, 1959).

(iv) All of those amino acids which either excited or depressed certain cholinoceptive cells affected non-cholinoceptive neurones in a similar fashion (CURTIS *et al.*, 1959; CURTIS, PHILLIS and WATKINS, 1960).

(v) The action of excitatory amino acids is antagonized by that of the depressant amino acids.

In regard to possible mechanisms, it appears certain from the limited structural variations allowable that only postulates based upon an initial attachment of the amino acids to membrane structures of specific shape, size, and charge distribution are tenable. The striking correlation between the structure–activity relationships of the two series of amino acids indicates a remarkably similar molecular morphology of the respective amino acid-receptors. The simplest and most likely postulate arising from this observation is that both the depressant and excitant amino acids act at the same sites, i.e. that the depressant amino acid receptor is the same, or part of the same, molecular component of the neuronal membrane as that acted upon by the excitant amino acids. With regard to the possible location of such receptors and their relationship to membrane sites concerned with normal synaptic transmissional processes, it may be considered that the membrane of neurones consists both of electrically excitable tissue, activity of which is responsible for the spike potential, and of 'transmitter receptor membrane' which is confined to the subsynaptic region. This latter membrane may itself be heterogeneous, consisting not only of components generally termed 'transmitter receptor sites,' which are chemically specific regions capable of combining with the transmitter, but also of closely related regions through which the permeability change appropriate for the synapse may actually be manifested.

The demonstration that γ-amino-*n*-butyric acid has no effect on the activity of presynaptic fibres (CURTIS *et al.*, 1959) suggests that this substance acts only upon the soma-dendritic regions of neurones. This may indicate that synaptic membrane is involved since synaptic knobs are confined to these portions of the neurone. From the evidence supporting the postulate that both series of amino acids have a common site of action, it follows that the excitant amino acids likewise may act only upon receptor membrane. It must be considered, however, that if these amino acids indeed act upon receptor membrane, this may be a consequence of their combination either with transmitter 'receptor sites' or with some other component of the receptor membrane.

Together with such consideration of the site of amino acid action, it is also necessary to consider the mode of action of these agents and whether they resemble transmitter substances. The excitant amino acids depolarize the membrane of all types of spinal neurone, not only non-cholinoceptive interneurones and motoneurones, but also cholinoceptive Renshaw cells (CURTIS, PHILLIS and WATKINS, 1960). Although the action upon the latter cells does not involve the 'receptor sites' specialized for combination with acetylcholine, since glutamate activation of these cells is not blocked by dihydro-β-erythroidine, acetycholine may not be the sole excitatory transmitter to Renshaw cells (CURTIS, PHILLIS and WATKINS, unpublished observations). Thus, other receptors on these cells, as well as those on interneurones and motoneurones, may be specialized for attachment of a transmitter related to the

excitant amino acids. However, it is considered that the probable absence of enzymic destruction as a factor terminating the action of these substances renders their relationship to transmitters unlikely. An action of the depressant amino acids resembling inhibitory transmitters in the mammalian spinal cord has already been excluded, the conductance change produced by those substances failing to alter the resting membrane potential of motoneurones (CURTIS *et al.*, 1959). This conductance change also excludes a curare-type block of excitatory synaptic action (cf. CASTILLO and KATZ, 1957) but provides a ready explanation of the diminution of both excitatory and inhibitory postsynaptic potentials by these compounds, because the currents generated by activated synapses, of either type, would be shunted through the low resistance regions of the membrane.

Two factors are probably involved in the antagonism manifested between members of the two series of amino acids when these are applied to the environment of neurones. The competition between excitant and depressant amino acids for the same receptor sites would diminish the activity of a given concentration of excitant amino acid in proportion to the number of sites now occupied by the depressant. In addition, those excitant molecules which do interact with membrane receptors would be unable to produce the same depolarization as in the absence of the depressant, since the particular membrane conductance change effected by the latter would tend to cancel potential changes produced elsewhere upon the membrane by reason of the current-shunting mechanism described above. This dual action of the depressant compounds probably accounts for the ability of a relatively low concentration of these amino acids to suppress the excitation of neurones evoked by a higher concentration of an excitant amino acid.

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Structural features of the amino acid receptor site

Before considering structural features of the postulated amino acid receptor site it is worthwhile to recapitulate the main conclusions reached by a study of the structure–activity relationships outlined in Tables 1 and 2. These may be summarized as follows: (i) one amino group and at least one acidic group is an absolute requirement for activity; these groups would of course be fully ionized in the physiological medium; (ii) with both the depressant and excitant amino acids, optimum activity is seen where the amino group is situated β- or γ- with respect to an acidic group; (iii) the additional acidic group of the excitatory amino acids is solely responsible for the particular permeability change effected by these acids compared with the depressants; this group is optimally situated α- with respect to the amino group; (iv) D- and L- forms of the one amino acid exhibit almost identical activities, at least in the cases of aspartic acid, glutamic acid, and α-alanine; (v) the introduction of substituent groups into the carbon chain or the amino group reduces or abolishes activity, depending on the size and number of the substituents. Any postulate concerning the mode of interaction of the amino acid with the neuronal membrane must take cognizance of these major features of the structure–activity relationships.

It is evident from these findings that activity is dependent upon interaction of the amino acid molecules with membrane structures of definite shape, size, and charge distribution, and that the membrane permeability change by which the action of an amino acid is mediated is the direct consequence of this transient interaction. The 'amino acid receptor' can thus be considered to consist of an arrangement of fully

ionic or partially charged atoms or groups located within a specific region of the molecular framework of the membrane, to which region, molecules in the extracellular fluid have only limited access.

It is possible to consider hypothetical receptor sites which involve either one, two, or three charged centres capable of interacting with ionized groups of the amino acids. As a first approximation, the forces involved in this interaction may be considered to be mainly electrostatic. A one-charge receptor may be regarded as unlikely, since it does not readily account for the fact that highest activity is associated with a definite optimum distance separating two ionic groups in an amino acid molecule. Hence the receptor can be assumed to possess at least two charges, these being a mean distance apart which approximates to the length of the GABA zwitterion under the particular conditions. The question arises, however, whether the excitant amino

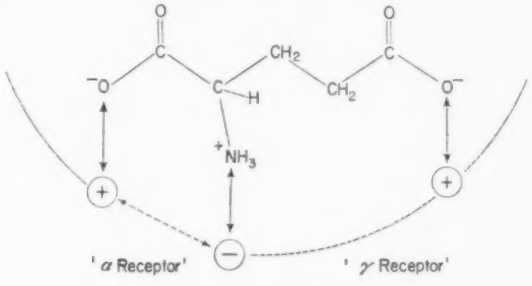

FIG. 1.—Three-point receptor (see text).

acids, with three charged groups, interact only with the same two receptor charges as the depressants, or whether there is yet a third receptor charge capable of an interaction with the additional ionic group of the excitant molecules. Such a three-charge receptor is schematized in Fig. 1 and the possible participation of just two, or, on the other hand, all three sites could perhaps account for the differential permeability change produced in the membrane by the depressant and excitant amino acids respectively. Within such a membrane region, the anionic site can again be envisaged to be separated from one of the cationic sites by a mean distance approximating that obtaining between the ionic terminals of GABA. The second cationic site must be situated closer to the anionic site than the first, and must, in fact, be no further from it than the maximal distance separating the amino and α-carboxylate groups of the molecules of glutamate, aspartate and cysteate, since these are the most active excitants. This proximity would suggest the possibility that these two sites actually interact with each other in the absence of an active amino acid. Such a receptor may be regarded as a composite of two receptors; one, the 'γ-receptor,' having greater affinity for amino acids in which the intercharge distance is close to that in γ-amino acids; the other, the α-receptor,' in which the intercharge distance is closer to that in α-amino acids. By this terminology, it is not necessarily implied that neutral α-amino acids, such as glycine and alanine, manifest depressant action by interaction with the α-receptor rather than with the γ-receptor, although such a possibility may exist, especially if the charged membrane sites are all fully ionic.

In the event of there being only two charged sites within the amino acid receptor, the excitant amino acids must be considered to interact with the membrane in precisely

the same way as the depressants (cf. Fig. 2), the additional carboxylate group causing the membrane depolarization by some mechanism which does not involve the participation of a third charged membrane site. Such a concept enables facile interpretation of the observation that D- and L-enantiomorphs of glutamate, aspartate, and α-alanine display similar activities, since a particular amino acid ion could probably adopt a multiplicity of positions in which electrostatic interaction (ionic bonding) between only two ionic groups and complementary groups upon the membrane would be possible. Ionic bonding, unlike covalent bonding, is not directional and could theoretically be manifested from any angle in the absence of steric hindrance. The D- and L- forms of an amino acid could thus be envisaged to adopt any of those positions in which projecting membrane atoms or groups did not sterically interfere with those of the amino acid. On the other hand, a three-charge receptor would

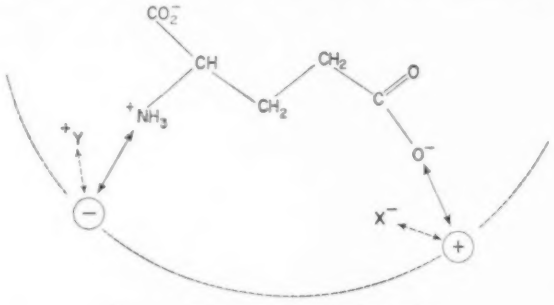

FIG. 2.—Two point-receptor (see text).

impose considerably more restriction on the positions available for excitant amino acid interaction with the receptor charges; nevertheless, it is again possible to account for the lack of stereospecificity shown by the receptor toward glutamic and aspartic acids. Thus in Fig. 1, one optical isomer of glutamate is depicted interacting with the three membrane charges so that the H atom of the asymmetric carbon atom is directed 'down' and the corresponding CH_2 group 'up' with respect to the plane of the paper: the other optical form must of necessity display a structure, during interaction with the receptor charges, in which the H atom is 'up' and the CH_2 group is 'down.' Some movement of the amino acid molecule would be possible during such interaction, but this would obviously be much less than with the two-point interaction schematized in Fig. 2, and the number of positions from which such interaction could be manifested would likewise be much less than with the two-charge receptor. On this concept, as again applied to glutamate for example, the fact that D- and L-forms have similar activities implies that there is sufficient space in the two directions ('up' and 'down' in Fig. 1) for the respective conformations of the $(CH_2)_2CO_2^-$ chain adopted in the two cases. This could most readily be explained on the hypothesis that the receptor charges project somewhat from the main plane of the membrane atoms in that region, and that the amino acid ion forms a bridge structure between them. In this case there may be adequate free space on both sides of the asymmetric carbon atom, particularly if the latter is only trisubstituted as in glutamate or aspartate, for each of the optical forms to interact with the receptor charges with equal facility.

The fact that substituent groups cannot be introduced into the molecules of active amino acids without considerable loss of activity indicates obstruction by

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membrane elements located either in the immediate vicinity of the receptor, or in more remote regions through which the amino acid molecules must pass to reach the receptor. This suggests that the receptor may actually be located within a 'cavity' of the molecular framework of the membrane. If this were so, in order for an amino acid to be active, not only must its charge distribution be complementary to that of the receptor site but also its effective 'width' must not exceed the narrowest region of the receptor cavity between the charged sites and the opening of the cavity into the external medium. In this respect the actions of N-methylaspartic acid (Table 1: 24) and N-methyl-β-alanine (Table 2: 28) are of interest. These two compounds had a longer duration of continued action, after cessation of their ionophoretic application, than had their non-methylated parent compounds, aspartic acid and β-alanine, respectively. On the other hand, as judged by the relative ionophoretic currents necessary to produce a particular intensity of action, the methylated derivatives appeared to be 'weaker' than the non-methylated compounds. Thus when the compounds were applied by equal currents, N-methylaspartic acid produced a lower frequency of spike response of interneurones than did aspartic acid. Similarly N-methyl-β-alanine was not as powerful a depressant of these cells as β-alanine. These results may indicate that the methylated compounds form 'amino acid–receptor complexes' of greater stability than those formed by aspartic acid and β-alanine, but that, for the same extracellular concentration of the methylated and non-methylated compounds, fewer molecules of the former actually penetrate to the active sites of the receptor because of some impeding influence of the N-methyl group.

Yet one other feature of the amino acid receptor merits discussion, this arising from the observation that depressant activity is exhibited over the wide range of homologous ω-amino acids, glycine to ε-aminocaproic acid, and excitant activity over the corresponding range, aminomalonic acid to α-aminopimelic acid. This is best explained on the hypothesis that, irrespective of whether the receptor be of two-charge or three-charge character, these charges are located upon molecular chains, the conformations of which are capable of variation. Changes in these conformations, resulting in differences in the distance between the charges, may be induced by the amino acid. It has earlier been suggested that, if the receptor be of a three-charge type, the charges are probably located on projecting structures. Hence it may be that the molecular chains on which the charges are now postulated to be located, are capable of adopting certain conformations in which they project out from the main plane of the molecular structure of which they are part.

The question arises how such two- or three-charge receptors can explain the observation that the zwitterions of neutral amino acids produce an increase in membrane conductance without depolarization whereas the anions of acidic amino acids depolarize the membrane. It is suggested that the charged sites of the receptor are located within pores or folds of the molecular structure of the neuronal membrane and that, under normal conditions, these sites control the ionic permeability of the region by an association with other charged entities, such as are represented by X^- and Y^+ in Fig. 2. These entities may be organic or inorganic ions which are freely diffusible when not 'complexed,' or they may be other components of the molecular structure of the membrane. The presence of an active amino acid in this region may be supposed to result in an interaction between the charged membrane sites and the corresponding ionized terminals of the amino acid (cf. Fig. 2), this interaction causing

the displacement of X^- and Y^+ and, in consequence, a change in the ionic permeability of the region. The actual permeability changes associated with amino acid action are as yet unknown but the most satisfactory explanation of the difference between the actions of acidic and neutral amino acids is that only the former result in the net entry of sodium ions into the cell, this accounting for the observed depolarization (cf. CURTIS, PHILLIS and WATKINS, 1960). The above postulate implies that both neutral and acidic amino acids displace X^- and Y^+ and that the difference in the resultant permeability changes is associated with the additional carboxylate group of the excitant amino acids. The role of this negatively charged group may be to mediate the transfer of cations, particularly sodium ions, directly from the extracellular to the intracellular phase by an 'ion-exchange' type of mechanism or perhaps to mediate this transfer indirectly by causing a critical disruption of the bonds complexing calcium ion with the membrane. This ion has been ascribed an important role in the control of membrane permeability (HODGKIN, 1958; MULLINS, 1959; cf. SHANES, 1958) and the reduction of its concentration in the extracellular fluid around spinal neurones leads to a depolarization which is probably caused by an associated depletion of 'membrane-bound' calcium (CURTIS, PERRIN and WATKINS, 1960). Direct mediation of cationic transfer by the additional carboxylate group in an ion-exchange type of action would imply that this group is not involved in interactions with the membrane, i.e. that the receptor is of the two-charge type as in Fig. 2. On the other hand, an interaction of the additional carboxylate group with membrane-bound calcium would essentially constitute a three-point amino acid–membrane interaction. In this case the cationic site of the 'α-receptor' of Fig. 1 would be specifically identified with membrane-bound calcium. Further, if the two charges of this 'α-receptor' interact with each other under normal conditions, this would suggest that Y of Fig. 2 may indeed be the same calcium atom, which, being only partially displaced from its firm association with the membrane by the amino group of the amino acid, could then become the cationic site of the α-receptor of Fig. 1: complete disruption of the calcium–membrane bonds and resultant cell excitation may then follow as a consequence of this action.

Correlation with related investigations

It remains to correlate the conclusions of this paper with those arising from other studies upon the same or related amino acids. The observations of PURPURA *et al.*, (1959) concerning the action of a large series of amino acids, including many of those represented in Tables 1 and 2, upon the surface potentials of the cerebral and cerebellar cortices, have been used as a basis for a classification of these substances. The amino acids have been divided into groups according to a postulated differential action upon excitatory and inhibitory synapses. In particular, it was postulated that ω-amino acids of a six-carbon chain length or less and ω-guanidino acids of a two- or three-carbon chain length selectively blocked excitatory postsynaptic potentials, whilst, on the other hand, longer chain ω-amino and ω-guanidino acids selectively blocked inhibitory postsynaptic potentials.

These postulates were based solely upon observations of changes in evoked surface potentials, no direct information concerning modifications in the specific processes of synaptic excitation and inhibition being obtained. The difficulties associated with the interpretation of extra-cellular records have already been discussed

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(CURTIS *et al.*, 1959), and it is suggested that the methods used by PURPURA *et al.* (1959) do not permit an analysis to be made in terms of the action of any substance upon the intimate processes of excitation of inhibition. This is evidenced by the recent observation that the short chain ω-amino acids depress both the excitation and inhibition of motoneurones and not the excitation process alone. It is probable that these agents affect cortical cells in the same way and the postulate that they are selective blockers of excitatory synapses therefore becomes untenable.

However, the results of PURPURA *et al.* (1959) regarding the effects of amino acids on the evoked responses of cerebral and cerebellar cortices can be compared with the present observations concerning the action of these substances upon field potentials within the spinal cord. A direct comparison can be made, for in both instances the potentials are generated by the activity of many cells, depression or

TABLE 5.—EFFECTS OF AMINO ACIDS ON FIELD POTENTIALS GENERATED BY GROUPS OF NEURONES

	ω-Amino monocarboxylic acids		ω-Guanidino monocarboxylic acids		Amino dicarboxylic acids
	Short chain ($<C_6$)	Long chain ($>C_6$)	Short chain ($<C_3$)	Long chain ($>C_3$)	
Spinal cord[1]	Depression	No action	No action	No action	Depression
Cerebellar[2] Cortex	Depression	No action	Depression	C_3–C_5, depression $>C_5$, no action	Depression
Cerebral[2] cortex	Depression	Augmentation	Depression	Augmentation	Depression

[1] Ionophoretic application through a microelectrode.
[2] PURPURA *et al.* (1959). Topical application.

blocking of which would produce a diminution in the recorded response. On account of the difficulty in interpreting the conversion of negative field potentials to positive potentials, these effects have been omitted from Table 5, which summarizes the results of the two investigations. The reduction in spinal field potentials produced by the excitatory amino acids has already been reported (CURTIS, PHILLIS and WATKINS, 1960). This was explained by the rapidity with which excess of excitatory agent leads to the inability of the affected membrane to support spike potentials, earlier excitation of individual cells being usually undetectable in an asynchronously affected population. The methods used by PURPURA *et al.* would not be expected to detect this early excitation.

Examination of Table 5 shows that there is good agreement between the results on the spinal cord and cerebellar cortex with all substances except the shorter chain guanidino acids. On the other hand, the cerebral cortex responses were modified by three other groups of substances, the shorter and longer chain ω-guanidino acids and the longer chain ω-amino acids, actions which were not paralleled in the spinal cord experiments. These differences may simply be a reflection of the low limit of concentration of the applied substance obtainable in a neurone population by the microelectrode technique. As with the method of topical application, the exact concentrations remain unknown, but the attainment of maximal concentrations in the vicinity of 3–11 $\times$ 10^{-3}M by ionophoretic application (cf. CURTIS, PHILLIS and

WATKINS, 1960; CURTIS, PERRIN and WATKINS, 1960) is favourable in the light of the statement (PURPURA *et al.*, 1959) that GABA is effective in concentrations of 10^{-4}M upon intravenous injection in preparations in which the permeability of the blood-brain barrier was altered. The ease with which Renshaw cells can be fired by ionophoretically applied acetylcholine (CURTIS and ECCLES, 1958) demonstrates that local concentrations of transmitter substances sufficient to affect receptors can be produced by this method. The possibility remains, nevertheless, that such concentrations are well below those obtained in neurone populations by the topical method of application. The higher concentrations produced by this latter procedure may not only affect neuronal membrane directly but also may indirectly alter neurone responses by an action upon other tissues or metabolic processes in the area.

A further difficulty in interpreting the action of a substance diffusing through nervous tissue is associated with the presence of enzymes within the tissue. The probable connection between the spreading depression produced by topical application of glutamic acid to the cerebral cortex (VAN HARREVELD, 1959) and the depolarization of spinal neurones by this amino acid has recently been pointed out (CURTIS, PHILLIS and WATKINS, 1960). In this regard the observation that D- and L- forms of glutamic and aspartic acids have approximately equal activity when applied ionophoretically to spinal neurones merits further attention, since VAN HARREVELD observed that D-glutamic acid has a lower threshold concentration for producing spreading depression than has the L-form. This difference would be satisfactorily accounted for if enzymes capable of disposing of D-glutamate were absent in the system, since, in spite of the topical application of equal concentrations of the two forms, a selective enzymic removal of L-glutamate would result in the D-form attaining a relatively higher concentration at the site of action. Under the experimental conditions of ionophoretic application, enzymic removal of the applied acids appears to be minimal (cf. also CURTIS, PHILLIS and WATKINS, 1960), and it is therefore unlikely that the apparent equivalence of D- and L- forms observed in these experiments, arises because the concentrations of the latter attained at the neuronal surfaces are lower than those of the former, despite equal rates of application. The difference in the results obtained by the two methods of applying amino acids is further exemplified by the observations that whereas L-glutamine and L-asparagine were inactive in the present investigation, both substances produced spreading depression when applied to the cortex, L-asparagine being more potent than L-glutamine (VAN HARREVELD, 1959; cf. also BURES, 1956; PURPURA *et al.*, 1959). A possible explanation of these and other discrepancies between the results obtained by ionophoretic and by topical application is that some substances applied topically to the cortex may be modified by enzymic action and that the observed effects in these cases are not due primarily to the added agent. An alternative explanation is that the cerebral cortex contains types of cells not represented in the spinal cord and that it is upon these cells or their synapses that the ω-guanidino amino acids, the longer chain ω-amino acids and other substances, found to be inert when applied ionophoretically to spinal neurones, manifest their action. Clarification of this point may follow the adaption of ionophoretic and intracellular techniques to experiments upon cortical cells.

Other substances having potent actions when applied topically to the cortex have been found to have less activity when applied ionophoretically to spinal cells. In a series of papers HAYASHI (1952, 1956, 1959*a*) has reported the effects of ω-amino acids

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upon cortical neurones, the observations being not incompatible with those of PURPURA *et al.*, (1959) and they can be similarly reinterpreted in the light of the present investigation. The suggestion, however, that γ-amino-β-hydroxybutyric acid is a more potent depressant than GABA (HAYASHI, 1959*b*) has not been confirmed (cf. Table 2: 20 and 3). Further, the finding that γ-aminobutyrylcholine is a more potent depressant of cortical activity than GABA (TAKAHASHI, *et al.*, 1959) is in contrast to the complete lack of action of this substance (cf. Table 2: 47 and 3) upon interneurones of the spinal cord.

Several recent investigations dealing with the actions of amino acids upon crustacean tissue are relevant to this discussion. EDWARDS and KUFFLER (1959) have reported an investigation upon the crayfish stretch receptor in which many of the substances of Table 2 were used. Since these could be applied at definite concentrations to this isolated tissue, the relative effectiveness of the different acids could be assessed. There was found to be a correlation between the depressant action of the neutral amino acids and their structure. In addition, some of the guanidino acids were nearly as effective as GABA. It was concluded that the mechanism of action of these acids resembled that of the inhibitory transmitter of these cells. However, since GABA also depressed certain lobster receptors not having inhibitory synapses (KUFFLER and EDWARDS, 1958) it is probable that, as also concluded in the present study, the action of these acids is unrelated to inhibitory synaptic action. ROBBINS (1959) analysed the effects of amino acids upon crustacean muscle, his results being similar to those of VAN HARREVELD (1959) and VAN HARREVELD and MENDELSON (1959). These investigations showed not only that the action of the amino acids was confined to post-synaptic structures but also that there was a relationship between the effect of an amino acid and its molecular structure. Thus, certain acidic amino acids, particularly L-glutamic acid, excited crustacean muscle whereas related neutral amino acids depressed synaptic excitation. There are some similarities between the structure–function correlations for the action of amino acids upon crustacean muscle, crustacean stretch receptors, and mammalian spinal neurones and presumably the observed differences in the susceptibility of these tissues are due to differences in the shape and disposition of the 'amino acid' receptors.

SUMMARY

(1) When applied ionophoretically to the external surfaces of spinal neurones, certain amino acids cause excitation of the nerve cells while others result in depression of neuronal activity.

(2) The most active of the excitatory amino acids studied were glutamic, β-aminoglutaric, aspartic, cysteic, and cysteine-sulphinic acids with β-hydroxyglutamic, N-methylaspartic, and N-formiminoaspartic acids also displaying strong activity. Amongst the depressant amino acids, β-alanine, γ-aminobutyric acid, and taurine were the most potent, followed by a group comprising N-methyl-β-alanine, γ-amino-β-hydroxybutyric acid, glycine, α-alanine, δ-aminovaleric acid, and β-amino*iso*-butyric acid.

(3) Investigation of a large number of related amino acids has led to the establishment of definite structure–activity relationships concerning these substances. Excitatory activity is associated with two acidic groups and one basic group; depressant action with one acidic and one basic group. Each of the excitatory amino acids corresponds

to an equally potent member of the depressant series in which the α-carboxyl group of the former is lacking.

(4) The optimum distance between the amino group and one of the acidic groups of the excitatory amino acids is two or three carbon atoms. The other acidic group is optimally situated α with respect to the amino group. The amino group is optimally primary. A sulphonic or sulphinic acid group may replace the β-carboxyl group in the case of a two-carbon separation of acidic functions.

(5) The optimum separation of the basic and acidic groups for depressant action is similarly two or three carbon atoms. The acidic function may again be sulphonic or carboxylic in the case of a two-carbon connecting chain.

(6) In both series, activity is abolished if the acidic or basic groups are not free and is reduced or abolished by substitution within the intermediate carbon chain or upon the amino group, depending on the size, number, and position of the substituents. Each of the two enantiomorphs of aspartic acid, glutamic acid, and α-alanine is approximately equally active.

(7) Possible sites of action of the amino acids upon the neuronal membrane are discussed together with some structural features of the postulated receptor sites and the possible mechanism by which the membrane permeability changes are manifested.

(8) The results are correlated with those of other investigators.

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GLUTAMIC-OXALACETIC TRANSAMINASE AND LACTIC DEHYDROGENASE ACTIVITIES IN BRAIN TUMOUR HOMOGENATES

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THE determination of glutamic-oxalacetic transaminase (GOT) and lactic dehydrogenase (LDH) activities in the cerebrospinal fluid (CSF) in diseases of the central nervous system has given the various authors who have investigated this problem variable and (especially in the case of brain tumours) dissimilar results. GREEN and co-workers (1957) found that the GOT activity in CSF was not significantly increased in any of the primary brain tumours they examined. In further research (GREEN *et al.*, 1958) on the CSF in 24 cases of brain tumours, among which was one sample of cyst fluid from an astrocytoma, the same authors reported that the GOT activity was raised in ten cases, but that such increase was significant only in three of them. FLEISHER *et al.* (1957) observed an increase of GOT in CSF from one case of astrocytoma and from one case of meningioma, and of the LDH in CSF from 2 cases of astrocytoma. These authors, in the same report, pointed out that there seemed to be no correlation between the degree of malignancy and the increase in the enzymes studied. THOMPSON *et al.* (1959) observed in the CSF of patients with brain tumours and other neurological diseases that most of the GOT and LDH values were within normal limits; the same authors, comparing the GOT, LDH and phosphohexose isomerase activities in normal brain tissue and in brain tumour homogenates, found no significant differences. GREEN and co-workers (1959) report that both GOT and LDH activities were greater in brain tumour than in normal human brain homogenates, on a fresh weight basis, but that these activities were much less per gram of soluble protein.

In our research the GOT and LDH activities have been studied in brain tumour homogenates in order to see whether or not these activities varied according to the histological type of tumour. Activities in neoplastic tissue have been compared with the activities of the same enzymes of normal brain homogenates. As the different composition of normal brain and brain tumour components might be a source of error, we thought of avoiding any such error, at least partly, by referring the data obtained not only to the wet weight, but also to the concentration per cent of the soluble protein.

MATERIALS AND METHODS

Ten pieces of normal human brain were obtained at operations exposing deep tumours, and histological examination of them verified that all were normal. Table 1 presents the values for the enzymic activities in these normal fragments.

Table 2 lists the enzyme activities from 25 brain tumours operated upon in our Institute. According to their histological type, they were found to comprise 8 glioblastomas, 2 ependymoblastomas 5 metastatic carcinomas, 4 ependymomas, 4 meningiomas, 1 acoustic neurinoma and 1 angioreticuloma.

All grossly apparent haemorrhagic and necrotic areas were removed from the tissue under

TABLE 1.—LDH AND GOT ACTIVITIES DETERMINED ON SOLUBLE PROTEIN BASIS IN NORMAL HUMAN BRAIN TISSUE

Normal brain tissue	LDH mean values per ml homogenate per 100 sec at 25°	GOT mean values per ml homogenate per min at 25°	Soluble protein mean values per ml homogenate
Gray matter (8 samples)	25 U	1·01 U	mg 5·3
Standard error	±1·67	±0·13	S.E. ± 0·01
White matter (3 samples)	14 U	0·75 U	
Standard error	±1·15	±0·23	

TABLE 2.—LDH AND GOT ACTIVITIES DETERMINED ON THE BASIS OF WET WEIGHT AND SOLUBLE PROTEIN IN DIFFERENT BRAIN TUMOURS

Case No.	Diagnosis	Soluble Protein mg/ml	LDH (a)	LDH (b)	GOT (a)	GOT (b)
1	Glioblastoma	12·0	35	15·9	0·39	0·18
2	Glioblastoma	5·6	29	27·4	—	—
3	Glioblastoma	13·5	60	23·5	2·60	1·02
4	Glioblastoma	6·3	43	36·1	2·07	1·74
5	Glioblastoma	—	50	—	0·96	—
6	Glioblastoma	14·7	52	18·7	1·35	0·48
7	Glioblastoma	6·5	57	46·4	1·44	1·17
8	Glioblastoma	—	33	—	0·39	—
9	Ependymoblastoma	—	71	—	2·10	—
10	Ependymoblastoma	8·2	51	32·9	1·44	0·93
11	Metastatic carcinoma (lungs)	5·7	71	66·0	0·57	0·53
12	(?)	14·3	68	25·2	0·60	0·22
13	(?)	10·7	38	18·8	0·58	0·28
14	(lungs)	—	45	—	0·64	—
15	(?)	—	34	—	0·87	—
16	Ependymoma	12·2	27	11·7	0·54	0·23
17	Ependymoma	5·2	27	27·5	1·32	1·34
18	Ependymoma	—	37	—	0·99	—
19	Ependymoma	6·9	40	30·7	1·05	0·80
20	Meningioma	9·5	37	20·6	1·11	0·61
21	Meningioma	6·1	41	35·6	1·20	1·04
22	Meningioma	—	32	—	0·38	—
23	Meningioma	11·7	21	9·5	0·39	0·17
24	Acoustic neurinoma	5·5	31	29·8	—	—
25	Angioreticuloma	5·6	29	27·4	—	—

(a) Units on fresh weight basis per ml of homogenate.
(b) Units on soluble protein basis.

examination and discarded. Then 2 g of it were put in 3 ml of a 0·1 M-phosphate buffer, pH 7·4, at 0° and were homogenized in a Potter-Elvehjem all-glass homogenizer. The homogenate was filtered after bringing to a dilution of one in ten. Two ml of the filtrate were used for the determination of the total nitrogen, according to the micro-method of Kjeldahl. Afterwards the filtered homogenate was once more suitably diluted with phosphate buffer.

GOT activity was determined by an adaptation of the method of KARMEN *et al.* (1955). 2·75 ml of $4{\cdot}2 \times 10^{-2}$ M-aspartic acid, 0·05 ml of $1{\cdot}2 \times 10^{-2}$ M-$DPNH_2$ and 0·05 ml malic dehydrogenase (0·5 mg per ml) were added to 0·2 ml homogenate, filtered and diluted as above. After shaking,

the cuvette containing this mixture was put into a water bath at 25°. After 15 min 0·1 ml of 0·2 M-α-keto-glutaric acid was added. After mixing, the solution was read at a wave length of 366 mμ at various time intervals. GOT activity was calculated in units according to LA DUE *et al.* (1954): a decrease in optical density of 0·001/min corresponds to 1 unit/ml (at room temperature = 25°).

The LDH activity was determined according to the method of WARBURG and CHRISTIAN (1936). To a 1 cm square cuvette were added 0·05 ml homogenate, filtered and diluted as described above, 2 ml of 5×10^{-3} M-pyruvic acid and 0·05 ml of 5×10^{-3} M-$DPNH_2$. After 3 min $DPNH_2$-oxidizing activity was read at 366 mμ. LDH activity was calculated according to BEISENHERZ *et al.* (1953). An enzymic unit represents a decrease in optical density of 0·100/ml/100 sec at room temperature (25°).

A Unicam Model SP 600 spectrophotometer was used for these readings.

RESULTS

Normal brain. The enzyme activities of normal human brain homogenates were very similar in each sample examined (Table 1). The mean values of the grey and white matter were considered as the normal ones.

Brain tumours. (a) *Results on fresh weight basis.* According to the histological diagnosis the LDH activity rises in the cases of glioblastoma (cases 3,4,5,6,7), metastatic carcinoma (cases 11, 12, 14) and in the two cases of ependymoblastoma. Less important variations, as compared with the normal mean values, were observed in the remaining malignant neoplasms and also in two ependymomas (cases 18, 19), in two meningiomas (cases 20, 21), in the acoustic neurinoma and in the angioreticuloma. The elevation of the GOT activity is much less remarkable. In fact, an increase of significance was found only in two glioblastomas (cases 3, 4) and in one ependymoblastoma (case 9). Values a little higher than the mean were observed in two other glioblastomas (cases 6, 7), in one ependymoblastoma (case 10) and in one ependymoma (case 17). GOT was never increased in the meningiomas. In nine cases (1, 8, 11, 12, 13, 14, 16, 22, 23) the GOT activity was found to be remarkably diminished. In only six cases out of the 25 examined were both LDH and GOT activities increased (cases 3, 4, 6, 7, 9, 10) and in three cases only (3, 4, 9) was this simultaneous increase remarkable.

(b) *Results on soluble protein basis.* The enzymic activity of brain tumour homogenates were also calculated in terms of the normal mean values of soluble protein expressed in mg per ml of brain tissue homogenates. The GOT activity was increased in two cases out of 15 (cases 4, 17) and in one of them (4) to a remarkable degree; the LDH activity was found to be elevated in six cases out of 18 (cases 4, 7, 10, 11, 19, 21), but only in two of them at all appreciably (cases 7, 11). Furthermore, in eight cases (1, 6, 11, 12, 13, 16, 20, 23) the GOT activity was found to be considerably lower than the mean values for the normal brain tissue homogenates. The LDH activity was remarkably diminished in three cases (1, 16, 23). Both enzyme activities were decreased in cases 1 and 16.

DISCUSSION

Such results are not easy to explain. All the tumours we examined, both benign and malignant, when compared with the normal brain tissue present a greater content of soluble protein. These results are in agreement with those obtained by GREEN *et al.* (1959). The increase in enzymic activities may be related to the greater concentration of soluble protein.

The reason why the GOT activity is decreased in many brain tumour homogenates

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with respect to its normal values, both when referred to the fresh weight and to the soluble protein, was not investigated. We merely note that this behaviour occurs, generally in malignant tumours.

The most important elevations in the LDH activity of brain tumour homogenates were observed only in malignant neoplasms, where a presumable increase in cell metabolism may be joined by important regressive and necrotic processes of the neoplastic tissue. The LDH activity of the cerebrospinal fluid ought to behave in a similar way. However, on comparing the results obtained by us in brain tumour homogenates with those obtained by the various authors who investigated the LDH activity of CSF, we can conclude that the activity of this enzyme in CSF is not related to the histological type of the tumour.

The GOT and LDH activities in CSF are now being studied in our laboratory.

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SUMMARY

(1) The GOT and LDH activities were examined in homogenates of 25 brain tumours. The values obtained were expressed with reference to the fresh weight of tissue and to the per cent concentration of soluble protein.

(2) When compared to values for normal brain the LDH activity was found to be increased in all the cases, but more noticeably in malignant tumours. The GOT activity was increased only in a few tumours, while it was decreased in most cases.

(3) The increased concentration of the soluble protein in neoplastic tissue homogenates may be related to the increased enzyme activity.

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THE QUANTITATIVE DISTRIBUTION OF 5-HYDROXY-TRYPTAMINE IN THE INVERTEBRATES, ESPECIALLY IN THEIR NERVOUS SYSTEMS*

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THE rather strange distribution of 5-hydroxytryptamine (5-HT, serotonin, enteramine) among a variety of cells and tissues, of both plants and animals, has been reviewed by COLLIER (1958). 5-HT occurs in such widely varying locations as blood platelets, mast cells, enterochromaffin tissue, venoms, the stinging nettle, and the vertebrate central nervous system. Add to these the banana and certain other fruits (UDENFRIEND, LOVENBERG and SJOERDSMA, 1959) and the situation is indeed confusing. Consequently, for those interested in 5-HT as another possible transmitter of nerve impulses, this unusual distribution, and probable multiple role of 5-HT, serves to divert attention from what may be the most important single function of 5-HT—that of a neurohumor.

Since the early evidence that 5-HT might serve as a cardioregulator in molluscs (BACQ, FISCHER and GHIRETTI, 1952; WELSH, 1953), there have been additional indications that it may play a transmitter role in certain invertebrates (WELSH, 1957). When it appeared that 5-HT was present in some invertebrate nervous systems in considerable amounts (FLOREY and FLOREY, 1954; WELSH, 1954) a survey of its distribution in the invertebrates was begun. Chromatography and bioassay were first employed in identifying and estimating 5-HT, but these procedures were superseded by the fluorescence method when a spectrofluorometer became available. We now have extracted and assayed tissues of a considerable number of the larger invertebrates, representing all of the major phyla. In a few species, various non-nervous tissues have been extracted for comparison with ganglia and nerves. In certain classes or phyla, where it is difficult or impossible to isolate nervous tissue because of small size (e.g. Platyhelminthes), or the diffuse nature of the nervous system (Coelenterata and Echinodermata), whole animals or pieces of animals have been extracted and assayed, first to determine whether or not 5-HT was present and then to attempt to learn something of its distribution within the organism.

Although there are many limitations to obtaining an adequate knowledge of the quantitative distribution of 5-HT in invertebrate nervous systems, this study, incomplete as it is, shows some interesting differences between phyla, for example between Arthropoda and Mollusca, and between classes within a phylum, as in the Mollusca. It is clear that 5-HT is found in primitive nervous systems and that its presence in the vertebrate central nervous system is only a recent part of a long phyloenetic history. If we knew the basic physiological roles of 5-HT in invertebrate

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nervous systems, we would have a better indication of its mode of functioning in man and the vertebrates.

METHODS

The majority of tissue samples came from living invertebrates which were dissected just before the tissues were to be extracted.

Sources of animals. The following animals came from laboratory cultures: *Pelomyxa* (*Chaos*)*, *Hydra*, *Daphnia*, *Artemia*, *Blaberus* and *Tenebrio*.

Gecarcinus were shipped from Bermuda; *Melongena*, *Fasciolaria* and *Busycon perversum* from Florida; scorpions from Arizona. All other living species were collected locally or obtained from commercial suppliers in New England.

A few tissues were dissected and placed in acetone or ethanol for shipment to Cambridge. These were from *Chiton*, *Octopus vulgaris* and *Panulirus* (Bermuda); *Aplysia*, *Octopus briareus* and *O. vulgaris* (Florida), and *Chelyosoma* (Puget Sound).

Preparation of tissues. Where possible, as in most molluscs and arthropods, nervous tissues and certain organs (e.g. heart or kidney) were dissected and cleaned under a dissecting microscope. They were kept at ice temperature until a sufficient sample was obtained (not over 3 hr) and then blotted carefully and weighed prior to homogenizing. In most species several animals were required to produce an adequate amount of a given tissue for assay, and particular ganglia, groups of ganglia, nerves or other tissues were pooled. The number of individuals utilized in each estimation is shown in Table 1 before the name of the tissue, organ or part.

Extraction procedure. The method of extracting 5-HT followed closely the analytical procedure as outlined by BOGDANSKI, PLETSCHER, BRODIE and UDENFRIEND (1956), with minor modifications made necessary by the small weights of tissue usually available. One of these modifications consisted of a change in the volume of 0·1 N-HCl in which tissue was homogenized. For all lots of tissue weighing less than 1·0 g, we homogenized in 2 ml of 0·1 N-HCl, while for lots weighing 1–7 g we used the ratio of 1 g tissue to 2 ml of 0·1 N-HCl.

After homogenizing with a motor-driven homogenizer, the entire sample was transferred to a 35 ml glass-stoppered centrifuge tube. (With *Asterias* and *Strongylocentrotus*, homogenizing was done in a Waring Blendor and extraction in a 100 ml flask.) The pH was adjusted approximately to 10 with solid Na_2CO_3, and 5 ml of borate buffer (pH 10) added. Next, 5 g NaCl and 20 ml of washed *n*-butanol were added, and the tube was shaken for 15 min. After centrifuging, the fluid was decanted into a separatory funnel, and the bottom (aqueous) layer discarded. The upper (butanol) layer was washed twice with borate buffer, and 30 ml of *n*-heptane and 3 ml of 0·1 N-HCl were added to the washed butanol phase. After shaking and allowing to separate, the entire acid layer was removed (volume slightly greater than 3 ml owing to removal of some water from the butanol phase) and 1 ml of conc. HCl added to produce a more characteristic fluorescence.

Identification and estimation of 5-HT *by its fluorescence.* At a pH of 4·0, and when activated at 295 mμ, 5-HT fluoresces maximally at 330 mμ (UDENFRIEND, WEISSBACH and CLARK, 1955). However, in 3 N-HCl the fluorescence of 5-HT shifts to a maximum near 550 mμ (UDENFRIEND, BOGDANSKI and WEISSBACH, 1955). These findings were utilized in the present study in the identification of 5-HT. The method has limitations since some other 5-hydroxyindoles in 3 N-HCl also fluoresce maximally near 550 mμ when activated at 295 mμ (e.g. bufotenine). However, after chromatographing extracts of nerve tissue of several species, only spots with R_F values corresponding with tryptophan and 5-HT were seen. The extraction procedure eliminates tryptophan from the sample that is read in the spectrofluorometer. Nevertheless, all values in Table 1 are presented as 5-HT equivalents as the possibility remains that some extracts contained a 5-hydroxyindole other than 5-HT.

A Farrand spectrofluorometer, based on the design of BOWMAN, CAULFIELD and UDENFRIEND (1955), was used in the current study. With the particular instrument employed, we obtained maximal excitation of 5-HT at a monochromator setting between 300 and 310 mμ and maximal fluorescence near 540 mμ (Fig. 1). The calibrations of the monochromators were checked with a mercury arc source, and, with repeated setting, they were found to be within $\pm$2–3 mμ of the expected wavelength. The differences between our observed maxima of excitation and fluorescence for 5-HT and those published by workers of the National Institutes of Health might be due to the emission spectrum of

* Full genus and species names will be found in Table 1.

the xenon arc lamp of the instrument we have used. The original lamp has been in use for over two years, and, in view of its satisfactory performance, there has been no opportunity to check the values that might be obtained with another lamp.

From the microammeter readings obtained with a given sample, those from a reagent blank were subtracted, and the value in 5-HT equivalents was read from standard curves. These curves were checked frequently by determinations on 5-HT standards. With the extraction procedure we

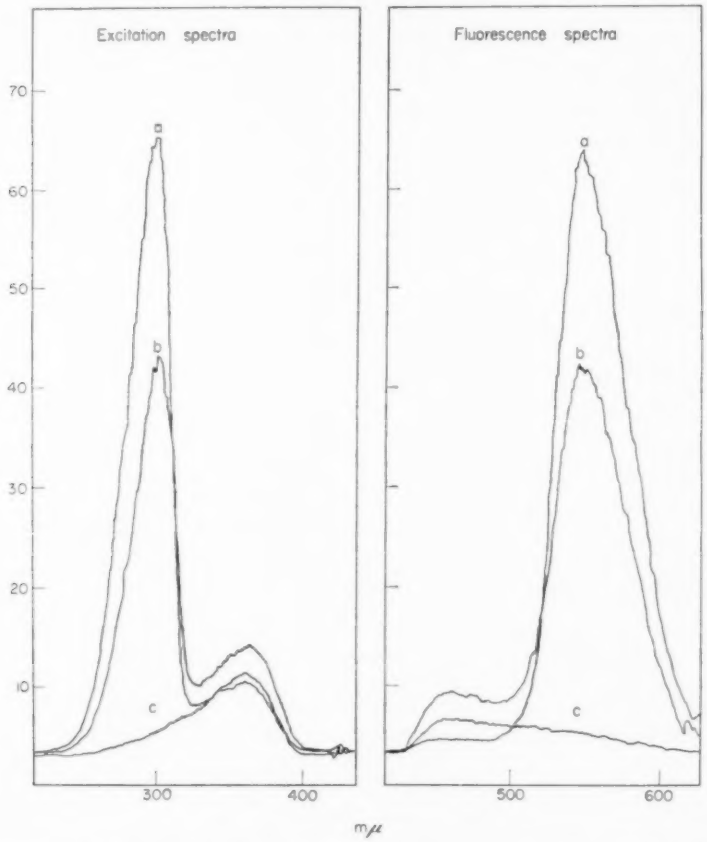

FIG. 1.—Typical records of uncorrected excitation and fluorescence spectra. (a) 5-Hydroxytryptamine 0·5 μg/ml in 3 N-HCl. (b) Extract from *Venus* ganglia—active material from 3·5 mg tissue/ml 3 N-HCl. (c) Reagent blank. Note that excitation peak of 5-HT and of the extract is near 300 mμ, while the characteristic fluorescence peak is in the visible near 540 mμ. The units of the vertical axis are arbitrary.

employed, some extracts contained large amounts of an unknown substance with a strong fluorescence near 450 mμ when excited at 305 mμ. This sometimes obscured the 5-HT fluorescence peak if the level of 5-HT was low. In such cases the excitation peak was often obvious, and the meter reading could be compared with those from standards to provide a satisfactory quantitative estimate of the 5-HT equivalents of the sample. Excitation and fluorescence spectra were usually recorded for later reference as in Fig. 1. Such records were especially useful when estimating very small amounts of 5-HT as they permitted more certain identification of excitation and fluorescence peaks.

The certainty with which a 5-HT-like substance was identified and quantitatively estimated depended on the amount of tissue available for extraction, its 5-HT content, and the presence of interfering substances in the final extract. In Table 1 some estimates will be found ranging from

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TABLE 1.—5-HYDROXYTRYPTAMINE (5-HT) IN INVERTEBRATES

Class and species	Tissue, organ or animal part	Weight (g)	5-HT (μg/g fresh tissue)
	PHYLUM PROTOZOA		
Class Sarcodina			
Pelomyxa carolinensis	(1500 estimated)	0·15	<0·02
	PHYLUM PORIFERA		
Class Demospongiae			
Halichondria panicea (Pallas)	colony	12·4	<0·03
	PHYLUM COELENTERATA		
Class Hydrozoa			
Hydra oligactis (Pallas)	several hundred, whole	0·38	1·50
Class Anthozoa			
Sagartia luciae (Verrill)	(10) whole	1·09	1·3
	(15) whole	1·46	0·74
Metridium senile L.	longitudinal half	2·52	0·04
	longitudinal half	2·49	0·05
	longitudinal half	2·57	0·05
	body wall	4·12	0·02
	body wall		0·05
	body wall	3·54	0·09
	oral disc and tentacles	3·98	0·08
	oral disc and tentacles	4·65	0·12
	oral disc and tentacles	4·02	0·17
	tentacles	1·69	0·24
	tentacles		0·47
	acontia	0·08	0·60
	acontia	0·23	0·97
	acontia	0·10	1·30
	PHYLUM PLATYHELMINTHES		
Class Turbellaria			
Dugesia tigrina (Girard)	(67) whole	0·078	2·0
	(75) in thirds head	0·160	2·5
	middle	0·232	1·3
	tail	0·174	1·7
	(100) in thirds head	0·031	3·3
	middle	0·044	1·6
	tail	0·014	3·6
Dugesia dorotocephala (Woodworth)	(50) whole	0·681	3·4
	(20) whole	0·205	1·6
	(50) whole	0·300	1·5*
	(45) in thirds head	0·146	1·4
	middle	0·169	1·4
	tail	0·131	4·3
	(50) in thirds head	0·158	1·8
	middle	0·229	1·6
	tail	0·172	3·7

* Venus heart bio-assay.

TABLE 1.—5-HYDROXYTRYPTAMINE (5-HT) IN INVERTEBRATES (*continued*)

Class and species	Tissue, organ or animal part	Weight (g)	5-HT (μg/g fresh tissue)
Dugesia sp.†	(75–100) anterior half	0·147	0·8
	(75–100) posterior half	0·123	1·1
Class Trematoda			
Pneumonoecis similiplexus (Stafford)	whole	0·08	<0·19
	PHYLUM NEMERTEA		
Lineus ruber (O. F. Müller)	(5) head	0·21	0·43
	(5) middle	0·62	0·18
	(5) tail	0·99	0·30
Cerebratulus lacteus (Leidy)	(2) head	1·24	2·9
	(2) middle	2·60	0·3
	(2) tail	3·76	0·08
	PHYLUM ANNELIDA		
Class Polychaeta			
Glycera dibranchiata (Ehlers)	(4) nerve cords	0·10	4·6
Arenicola marina (L.)	(4) nerve cords	0·75	3·1
	(1) nephridial tissue	0·03	<0·14
	(1) body wall	1·20	2·3
Amphitrite ornata (Leidy)	(6) nerve cords	0·13	5·4
	(6) tentacles	3·07	0·08
	(6) gills	1·21	0·26
	(1) body wall	1·63	0·19
Class Oligochaeta			
Lumbricus terrestris (L.)	(5) nerve cords	0·133	10·4
Class Hirudinea			
Hirudo medicinalis (L.)	(5) nerve cords and sinus sheaths	0·043	6·9
	PHYLUM SIPUNCULOIDEA		
Golfingia(Phascolosoma) gouldii	(6) nerve cords	0·033	1·8
	(8) cephalic ganglia and nerve cords	0·071	4·7
	PHYLUM MOLLUSCA		
Class Amphineura			
Chiton tuberculatus L.	nerve tissue	0·067	17·5; 10·0*
Class Pelecypoda			
Pecten magellanicus (Gmelin)	(24) visceral ganglia	0·089	36·0
Mytilus edulis (L.)	(20) cerebropleural ganglia	0·022	15·0
	(20) pedal ganglia	0·017	15·0
	(18) visceral ganglia	0·021	10·0

* Venus heart bio-assay
† Probably Dugesia; from Oregon.

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TABLE 1.—5-HYDROXYTRYPTAMINE (5-HT) IN INVERTEBRATES (*continued*)

Class and species	Tissue, organ or animal part	Weight (g)	5-HT (μg/g fresh tissue)
Artica islandica (L.)	(16) ganglia†	0·152	20·0
Venus mercenaria (L.)	ganglia (26 determinations over 16 months)		mean 40·0
Spisula(= *Mactra*)*solidissima* (Dillwyn)	(3) ganglia	0·044	8·0
	(3) ganglia	0·040	14·3
	(3) connectives	0·073	2·2
Ensis directus (Conrad)	(6) ganglia	0·032	31·0*
	(5) ganglia	0·047	21·0
	(10) ganglia	0·061	39·0
Mya arenaria (L.)	(12) ganglia	0·028	22·0
Anodonta cataracta Say	(1) kidneys	0·373	1·3
Class Gastropoda			
Polynices heros (Say)	(6) ganglia and nerves	0·073	2·6
	(6) ganglia and nerves	0·093	2·6
Buccinum undatum (L.)	(8) ganglia	0·170	7·7
Melongena corona (Gmelin)	(3–6) ganglia (4 determinations)	0·053 mean	4·9–11·0 mean 9·0
	(3) kidneys	1·02	0·63
	(3) kidneys	0·89	0·96
Busycon canaliculatum (L.)	ganglia (4 determinations)	0·11 mean	8·4–9·7 mean 9·2
	(1) nerves	0·12	2·5
	(1) nerves	0·17	2·0
	ganglia and nerves (7 determinations)	0·14 mean	4·3–8·5 mean 6·1
Busycon perversum (L.)	(2) ganglia and nerves	0·15	3·7
Fasciolaria tulipa (L.)	(2) ganglia and nerves	0·11	7·6
	(2) ganglia and nerves	0·07	9·4
	(2) kidneys	2·23	1·04
	(2) kidneys	2·22	1·7
Planorbis planorbis L.	several (minus shell)	5·2	0·09
Thais lapillus (L.)	(5) minus shell, liver and kidney	3·53	0·44
	(17) hypobranchial glands	0·47	0·01

* Venus heart bio-assay.

† In this class, unless otherwise indicated, the term 'ganglia' includes the three kinds: cerebropleural, pedal, and visceral.

TABLE 1.—5-HYDROXYTRYPTAMINE (5-HT) IN INVERTEBRATES (*continued*)

Class and species	Tissue, organ or animal part	Weight (g)	5-HT (μg/g) fresh tissue
Class Cephalopoda			
Loligo pealii (Lesueur)	(3) 'brain'	0·35	0·7
	(3) optic ganglia	1·15	1·15
	(4) median salivary gland	0·17	0·38
	skin	2·05	<0·04
Octopus briareus (Robson)	(1) 'brain', optic and stellate ganglia	0·52	1·6
Octopus vulgaris L. (Florida)	(1) 'brain', optic and stellate ganglia	0·62	3·2
Octopus vulgaris L. (Bermuda)	(1) 'brain'	0·52	0·8
	(1) 'brain' and stellate ganglia	0·26	0·56
	(1) optic ganglia	0·92	2·3
	(1) optic ganglia glands	0·40	1·0
	(1) ant. salivary glands	0·59	2·4
	(1) ant. salivary glands	0·58	0·48
	(1) post. salivary glands	0·67	72·0
	(1) post. salivary glands	0·70	68·0
	PHYLUM ARTHROPODA		
Class Crustacea			
Daphnia magna (Straus)	many, whole	1·5	0·02
Artemia salina (L.)	many, whole	1·98	0·10
Orconectes virilis (Hagen)	(24) nerve cords	0·58	<0·10
	(5) green glands	0·57	<0·097
Homarus americanus (Milne-Edwards)	(3) nerve cords	0·80	<0·02
	(3) nerve cords	0·81	<0·03
	(3) leg nerves*	0·99	<0·01
	(3) green glands	2·47	<0·06
Panulirus argus (Latreille)	nerve cord	0·27	<0·10
Cancer irroratus (Say)	(12) ventral ganglia	1·69	<0·03
	(10) ventral ganglia	0·88	<0·05
	(12) ventral ganglia	1·14	<0·03
	(10) ventral ganglia	1·87	<0·07
	(10) 'brain'	0·19	<0·11
	green glands	0·41	1·0
	(4) green glands	0·24	0·31

* Pooled leg nerves from the number of animals indicated in parentheses.

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TABLE 1.—5-HYDROXYTRYPTAMINE (5-HT) IN INVERTEBRATES (*continued*)

Class	Tissue, organ or animal part	Weight (g)	5-HT (μg/g fresh tissue)
Cancer borealis (Stimpson)	(3) ventral ganglia	0·40	<0·06
	(8) ventral ganglia	1·66	0·08
	(8) ventral ganglia	1·36	0·02
	(7) ventral ganglia	2·15	0·02
	(3) leg nerves*	0·60	<0·04
	(10) 'brain'	0·14	0·08
	(16) pericardial organs	0·061	3·2
	(12) pericardial organs	0·079	2·6
	(12) pericardial organs	0·043	4·0
	(4) green glands	0·40	0·72
Carcinus maenas (L.)	(13) ventral ganglia	0·38	<0·04
	(13) leg nerves*	0·61	<0·03
	(12) pericardial organs	0·027	2·8
	green glands	0·17	<0·12
Gecarcinus lateralis Frem.	(3) ventral ganglia and leg nerves	0·28	<0·15
	(3) claw muscles	2·18	<0·02
	(3) green glands	0·11	0·80
Class Arachnoidea			
Limulus polyphemus (L.)	(1) heart and cardiac ganglion	1·87	0·09
	(1) intestine	1·75	0·09
	(1) nerve cord	0·78	0·20
	(1) nerve cord	0·89	0·10
	(1) leg nerves*	0·46	0·29
	(1) leg nerves*	0·44	0·15
	(1) coxal glands	0·56	0·80
Hadrurus arizonensis (Ewing)	(1) sting segment	0·12	—
	(1) sting segment	0·11	<0·55
	(1) abdominal segments (second and third)	0·18	—
	(1) head and thorax (without digestive gland)	0·57	<0·12
Vejovis spinigerus (Wood)	(1) sting segment	0·003	—
	(1) sting segment	0·005	—
Vejovis sp.	(1) sting segment	0·007	138·0
	(1) sting segment	0·004	50·0
Class Insecta			
Blaberus gigantea	(10) nerve cords	0·16	<0·02
	(12) heads and first thoracic segments	3·66	0·05
Tenebrio molitor	(24) whole larvae	3·17	0·05

* Pooled leg nerves from the number of animals indicated in parentheses.

TABLE 1.—5-HYDROXYTRYPTAMINE (5-HT) IN INVERTEBRATES (*continued*)

Class and species	Tissue, organ or animal part	Weight (g)	5-HT (μg/g fresh tissue)
Sceliphron caementarium (Drury)	(11) heads	0·10	0·47
	(11) thorax	0·47	0·19
	(11) abdomens	0·25	29·0
Polistes sp.	(15) abdomens	0·59	34·4
Apis mellifera	(85) heads	0·83	0·07
	(68) heads	0·61	0·16
	(85) tips of abdomens	1·00	1·3
	(68) tips of abdomens	0·45	3·2
	(21) venom apparatus	0·03	21·0
	PHYLUM ECHINODERMATA		
Class Asteroidea			
Asterias forbesi (Desor)	(1) gonads removed	42·0	0·016
Class Echinoidea			
Strongylocentrotus drobachiensis (O. F. Müller)	(1) gonads removed	12·0	0·03
	(1) gonads removed	55·0	0·01
Class Holothuroidea			
Synapta inhaerens (O. F. Müller)	whole, in acetone	10·0	0·04
	whole, fresh	2·5	0·02
Thyone briareus (Lesueur)	(2) region of nerve ring	0·68	0·05
	(2) region of nerve ring	0·78	0·03
	PHYLUM CHORDATA		
Class Ascidiacea			
Molgula manhattensis (DeKay)	(6) parts of body wall, including some nervous tissue	0·68	<0·11
	(3) minus testes and body fluid	1·42	<0·07
	(8) intestine	0·29	<0·17
	(7) kidney	0·09	<0·43
Chelyosoma productura	(300+) ganglia in acetone	—*	0·3†

* Weight not known.
† Total 5-HT in sample.

'less than 0·01 μg/g' to 'less than 0·44 μg/g' when positive identification and quantitative estimation were impossible for one or more of the above-mentioned reasons. With an abundance of tissue and little interfering substance, values in the range of 0·01 to 0·10 μg 5-HT per gram of fresh tissue were readily determined.

The values of 5-HT given in Table 1 are expressed in terms of μg per g of fresh tissue. No corrections have been made for failure of the method to extract all of the 5-HT from a tissue. Since the mean recovery of added 5-HT was 75 per cent, it may be assumed that the estimates as given are too low by an amount equivalent to one-third of the value. For a comparison with values to be found in the literature, based on the weight of dried tissues, the values reported herein must be multiplied by a factor which would ordinarily lie between 5 and 10, since the dry weights of most invertebrate tissues are between 10 and 20 per cent of their wet weights.

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RESULTS AND COMMENTS

Whole animals or tissues from over 60 species, representing 11 phyla, have been treated by the extraction procedure and their content of 5-HT-like substance has been estimated. In many instances, several determinations have been made on a given tissue. Although the principal aim was to examine nervous systems for the occurrence and quantitative distribution of 5-HT, many determinations were made on whole animals or parts of animals (Table 1). The weight of the tissue, organ, or part is shown to indicate that, where values are stated to be less than a given amount, failure to detect 5-HT was sometimes due to the extraction of too small a weight of tissue. In almost all instances the 5-HT estimates have been calculated in terms of μg per g of fresh tissue.

Comments on the results given in the Table will be of four main types: (a) of the nature of footnotes, (b) comparisons between phyla, classes, and species, of the 5-HT levels of a given tissue, (c) comparisons between tissues of a given species, and (d) comparisons with the results of other workers. Comments will be grouped by phyla and will follow the order given in Table 1.

Protozoa and Porifera

Only one species from each of these phyla was examined. No 5-HT was detected in the giant amoeba, *Pelomyxa*, nor in the bread-crumb sponge, *Halichondria*, by the method employed. We know of no other determinations made on representatives of these two phyla. It is important that others be made as these are the only two phyla where neurons are absent.

Coelenterata

The diffuse nature of the primitive nervous system of this group makes it difficult, if not impossible, to separate nerve tissue from surrounding tissues. Extracts were made of entire individuals or groups of individuals of three species, representing two classes. One lot of *Hydra oligactis*, a common brown hydra, gave a value of 1·5 μg 5-HT/g fresh tissue. Hydra is the most primitive animal in which we have found 5-HT, and its content of this amine is higher than that of almost any other whole animal that we have examined. Values for whole planaria are rather similar, while those for certain wasps would be considerably higher if whole animals were extracted. High wasp values would result from the very large amounts of 5-HT in their venom apparatus. Two lots of a small sea anemone, *Sagartia luciae*, yielded 5-HT values only a little lower than the hydra value, but the sea anemone, *Metridium*, was found to contain very little 5-HT. When different parts of *Metridium* were extracted separately, those parts containing large numbers of stinging organelles or nematocysts (e.g. tentacles and acontia) were found to yield much more 5-HT than body wall. This observation has been reported elsewhere (WELSH, 1960) and suggests that 5-HT may be a constituent of coelenterate venom as it is of many other venoms.

Earlier reports of 5-hydroxyindoles in coelenterate tentacles and nematocysts were those of WELSH (1956) and PHILLIPS (1956). These observations were extended by MATHIAS, ROSS and SCHACHTER (1957, 1958) who reported that of four species of sea anemones examined, significant amounts of 5-HT were found only in *Calliactis parasitica* (a species earlier studied by one of us). Since, in *Calliactis*, MATHIAS *et al.*

(1958) found extremely large amounts of 5-HT in 'tissues of the coelenteric cavity' (500–600 μg/g freeze-dried tissue) and considerably less in tentacles (5–15 μg/g) and acontia (20 μg/g) they conclude that it is unlikely that 5-HT is associated specifically with nematocysts. This may well be true if they mean that 5-HT is not *restricted* to nematocysts. It would be of much interest to know in which parts the large amount of 5-HT in *Calliactis* coelenteric tissue is actually located. Also, it will be of interest if a coelenterate can be found that will yield nerve tissue at least relatively free of surrounding tissue. In this way one might learn if the most primitive of all nervous systems contains 5-HT or a related amine. ROSS (1957) finds tryptamine more effective than 5-HT in facilitating certain motor responses of *Metridium* and *Calliactis* muscle, while both of these indole amines are more active than other common amines.

Platyhelminthes and Nemertea

The flatworms are the most primitive animals that show marked cephalization of the nervous system. Sense organs and ganglion cells are concentrated in the head region, while two pairs of longitudinal nerves run the length of the body giving off lateral branches and connectives. If any of the known transmitter agents (neurohumors) are present in the flatworm nervous system they should be found in greatest abundance in the head region where synapses are most concentrated. One of us (WELSH, 1946) has shown that *Planaria maculata* (= *Dugesia tigrina*), the common brown planarian, has a much higher level of acetylcholine in the anterior third (6 μg Ach/g tissue) than in the middle third (1·6 μg/g) or in the tail third (1·2 μg/g). BULLOCK and NACHMANSOHN (1942) found planaria to have unusually high cholinesterase activity. With these results in mind we were prompted to look for 5-HT in head, middle and tail portions of planaria. One lot of whole brown planaria gave a value of 2 μg 5-HT/g. The first lot of 75 brown planaria (local) which were cut in thirds gave values averaging 1·9 μg/g (Table 1). More 5-HT was found in the head than in the middle or tail thirds. However, another lot of much smaller planaria from North Carolina (purchased as *Dugesia tigrina*) yielded values for head and tail that were approximately equal and about twice the value for the mid region.

One lot of 50 unusually large black planarians, *Dugesia dorotocephala*, gave a higher 5-HT value than two other lots from a different source. The extract of one of these two lots was assayed with the spectrofluorometer, the other with the *Venus* heart assay. Such very close agreement between the values obtained by the two methods is not always achieved.

Two lots of black planaria were cut in three parts and the pooled head, middle and tail pieces were treated separately. The tail thirds of both lots gave considerably higher values than did the other parts. This was quite unexpected and the reason for it is not yet clear. After the observations on these black planaria were made, we found several species of invertebrates whose excretory organs contained relatively large amounts of 5-HT. We believe it may sometimes be excreted intact rather than converted to 5-hydroxyindole acetic acid as in mammals. Some fresh water turbellarians have a more complex nephridial apparatus than others (KROMHOUT, 1943). Conceivably there is a high content of 5-HT associated with the excretory systems of such species (see General Discussion).

A species of planarian sent from Oregon divided spontaneously into approximately equal anterior and posterior halves soon after arrival. Slightly more 5-HT

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was found in the posterior half. Further work is necessary before the relation of 5-HT to specific structures in the free-living flatworms is known.

One lot of frog lung-flukes (*Pneumonoeces*) from *Rana pipiens* was analysed. If 5-HT was present, it was below the level that could be detected (the weight of tissue was small). MANSOUR, LAGO and HAWKINS (1957) used the rat uterus to demonstrate the presence of a 5-HT-like substance in extracts of the parasitic trematode, *Fasciola hepatica*, and MANSOUR (1957) found muscle of the liver fluke to be stimulated by small doses of 5-HT.

The Nemertea or ribbon worms have a distribution of nerve tissue similar to that found in the flatworms. Ganglia are restricted to the head region, but two pairs of nerve trunks with lateral connectives run the length of the body. Two common Atlantic Coast species were divided into thirds and analysed. Heads of *Lineus ruber* gave a slightly higher value than tails, with least in the mid-region. One specimen of the much larger *Cerebratulus* gave a value for the anterior portion nearly ten times as great as that for the mid-region and over 30 times that for the tail. The values agree rather well with the proportional distribution of nerve tissue.

If 5-HT is located, at least in part, in the nervous systems of flat worms and ribbon worms, it must be present in rather large amounts to yield such a high value as that, for example, obtained for the head region of *Cerebratulus* where the nerve tissue makes up such a small portion of the total weight.

Annelida and Sipunculoidea

The nervous system of the annelids, consisting of a chain of ganglia, extends the entire length of the body and is often imbedded in the muscle of the ventral body wall, Its removal, free of surrounding, non-nervous tissue is difficult. While it can be accomplished quite satisfactorily in the common earthworm, *Lumbricus*, its removal is well-nigh impossible in a species such as *Arenicola*. Nevertheless, some annelids proved to be the most primitive animals available to us from which nerve tissue could be isolated free of non-nervous tissue and in amounts needed for 5-HT determinations. It was of much interest, therefore, to find that the annelid nervous system contains appreciable amounts of this indole alkyl amine. Two species of marine polychaetes (*Glycera* and *Amphitrite*) gave values of about 5 μg/g nerve cord. *Arenicola* cords were surrounded by so much muscle and other tissue that the value given is for mixed muscle, nerve, and other tissue and, in fact, is only a little greater than that for body wall (dorsal and lateral). Values for a few other non-nervous tissues of *Arenicola* and *Amphitrite* are given in Table 1.

Five nerve cords of the common earthworm were dissected, cleaned, and assayed. They gave a value of 10·4 μg/g tissue. Five cords of the European medicinal leech, *Hirudo medicinalis*, were still surrounded by their sinus sheaths when weighed. If the 5-HT is confined to the nervous tissue the value of 6·9 μg is somewhat low. CORREALE (1956) reports that ERSPAMER, using bioassay and chromatography, found less than 0·05 μg 5-HT/g of ventral ganglionic chain of *Hirudo medicinalis*. Since we have values for cephalopod and arthropod nervous tissues that are in good agreement with those of ERSPAMER, as reported by CORREALE, there is reason to question his failure to find 5-HT in *Hirudo* ganglia.

The nervous system of the sipunculoid worm, *Golfingia* (= *Phascolosoma*) is easily removed. The entire nervous system including cephalic ganglia, circumpharyngeal

connectives, and cord gave 2·5 times as much 5-HT as cord alone. This is probably related to the distribution of cell bodies and synapses.

It will be pointed out later on in this paper that the only other animals with amounts of 5-HT in their nervous systems comparable to those found in the annelids and their close relatives, are the molluscs.

Mollusca

With the recent independent discovery of two species of *Neopilina*, the most primitive living molluscs, with many annelid-like features, the phylogenetic relationship between annelids and molluscs is now more firmly established. Therefore, one might expect to find closer biochemical affinities between annelids and molluscs than between, say, annelids and arthropods. It was not surprising, therefore, to find large amounts of 5-HT in the nervous systems of most molluscan species. Representatives of four of the six existing classes were procurable for study. The relatively large size of many molluscs and the ease with which ganglia and connectives can be removed encouraged the examination of a considerable number of species, especially from the classes Pelecypoda and Gastropoda. These two classes provide highly satisfactory experimental material for both *in vivo* and *in vitro* studies of 5-HT formation, storage, release, and action (WELSH and MOORHEAD, 1959*a*, *b*; WELSH, 1958, and unpublished). Therefore, it is of particular importance that we have some knowledge of the quantitative distribution of 5-HT within their nervous systems.

Class *Amphineura:* The nervous system of this primitive group consists of a circumoesophageal nerve ring with two pairs of longitudinal nerves running from it. Nerve cells are scattered throughout the system, and ganglia with their concentrated synaptic regions are lacking. A small sample of this nervous tissue of only one species, *Chiton tuberculatus*, was available. A fraction of this sample was assayed on the *Venus* heart and gave a value of 10 μg/g. The remainder was used for a fluorescence determination and gave a value of 17·5 μg/g. The discrepancy between the two values resulted, in part, from the small amount of tissue available (67 mg) and the limited number of comparisons with known 5-HT that could be made in the bioassay.

Class *Pelecypoda:* Nervous tissues from seven species of large marine molluscs of this class were assayed. From the sea scallop, *Pecten magellanicus*, only the visceral ganglia were removed; they had an estimated 5-HT content of 36 μg/g. The three types of ganglia of the edible sea mussel, *Mytilus edulis*, were separately pooled and assayed. The cerebropleural and the pedal ganglia yielded identical values (15 μg/g). The lower value for the visceral ganglia (10 μg/g) is perhaps due to the difficulty of removing and cleaning these ganglia from *Mytilus*. A small amount of adhering non-nervous tissue results in an abnormally low value since most molluscan tissues, other than nerve, contain little 5-HT (WELSH and MOORHEAD, 1959*a*). Having observed no consistent differences in the 5-HT levels of the different ganglia of *Venus*, estimations on this and other species were made on pooled ganglia of the three types. In this section of the Table the number in parentheses refers to the number of individuals from which the sets of ganglia were obtained.

We have made more estimates of 5-HT in *Venus* ganglia than in nervous tissue of any other species. In a preliminary report (WELSH and MOORHEAD, 1959*a*) we gave a mean value of 30 μg/g for 11 determinations. Since some of the earlier values were low, owing perhaps to less proficient dissection and cleaning, we now report a mean

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of 40 μg/g, based on 26 determinations extending over a 16 month period. This is one of the highest values obtained for any nervous tissue, and equivalent amounts of 5-HT of nervous origin are found only in other bivalve molluscs. It was fortuitous, but fortunate, that the earliest attempt to demonstrate 5-HT in an invertebrate nervous system by means of paper chromatography was made with *Venus* ganglia (WELSH, 1954). Levels of 5-HT in a variety of tissues of *Venus* have been reported earlier (WELSH and MOORHEAD, 1959*a*). No organ or part, outside the nervous system, was found to contain more than 0·75 μg/g (mantle edge), and it was suggested that this 5-HT might have come from nerve endings. No 5-HT was detectable in the blood of *Venus*.

The large size of the surf clam, *Spisula*, allowed the removal of enough of the connectives between ganglia for a 5-HT determination. It is of interest that these tracts of axons gave a 5-HT value of only 2·2 μg/g, while two lots of ganglia gave an average of 5 times this amount. This suggests that the 5-HT is concentrated in cell bodies or synaptic regions.

Ganglia of *Ensis*, the razor clam, and of *Mya*, the soft-shelled clam, contain relatively very large amounts of 5-HT, and it might be noted that *Ensis* ganglia are easy to locate, remove, and clean.

Class *Gastropoda:* In this class most of the ganglia are grouped around the anterior end of the oesophagus and are linked by short connectives in such a manner that their removal is not difficult. Nerves run from these ganglia to all parts of the body. Varying lengths of nerves were sometimes left attached to the ganglia and in such instances this is noted in the Table by 'ganglia and nerves'. When the nerves were cut close to the ganglia only the word 'ganglia' is found. The 5-HT content of ganglia of three species of marine gastropods (*Buccinum*, *Melongena*, and *Busycon canaliculatum*) is quite uniform (7·7, 9·0, and 9·2 μg/g). Nerves of *B. canaliculatum* have a level of 5-HT about one-fourth that of the ganglia of this species. If this is generally the case in the gastropods, as seems likely, values for 'ganglia and nerves' would be lower than for 'ganglia'. This was demonstrated to be true for *Busycon*.

5-HT determinations on a variety of tissues of *Busycon canaliculatum* have been made and reported earlier (WELSH and MOORHEAD, 1959*a*). Considerable amounts of 5-HT were found in the kidney (1·4 to 2·3 μg/g). Kidneys of *Melongena* and *Fasciolaria* were also extracted and found to contain relatively large amounts of 5-HT (see General Discussion).

The hypobranchial gland of certain members of the Muricidae (e.g. *Murex*) metabolizes tryptophan to a variety of indole derivatives, including Tyrian purple. ERSPAMER (1954*a*) found a high level of 5-HT (80–290 μg/g) in the hypobranchial gland of *Murex trunculus*. We studied this gland in *Thais lapillus*, a member of the same family, but could detect no 5-HT in the extract. However, an extract prepared from *Thais* with shell, liver, and kidney removed contained an appreciable amount of 5-HT (0·44 μg/g).

Class *Cephalopoda:* Nervous tissues from the common squid, *Loligo*, and two species of *Octopus* were assayed. Living squid were available to us, but the *Octopus* tissues had been preserved in alcohol or acetone. An *Octopus* tissue with its solvent was dried, and the residue was treated with 0·1 N-HCl. We then proceeded to partition as with fresh tissue. In this section of Table 1, the term 'brain' is only a convenient way of identifying the collected ganglia of the head region other than the large optic

ganglia. All values are expressed in terms of the fresh tissues which were weighed before placement in alcohol or acetone.

The most interesting finding from this class of highly organized and very active molluscs is the relatively small amount of 5-HT in the nervous system. In the instances where optic ganglia were extracted separately, they contained slightly more 5-HT than the other pooled ganglia. However, the maximal amount of 5-HT found in any ganglion or group of ganglia was the value (3·2 μg/g) for the pooled ganglia of one specimen of *Octopus vulgaris*, dissected for us by colleagues in Florida. The range of values for these cephalopod ganglia (0·7 to 3·2 μg/g) is significantly lower than that for comparable tissues of any of the other classes of molluscs. It is of much interest that the least active and specialized molluscs, the pelecypods, have far more 5-HT in their ganglia than do these specialized cephalopods, while the gastropods are intermediate in respect both to specialization and level of ganglionic 5-HT.

Our values for 5-HT levels in various groups of ganglia of *Octopus vulgaris* (0·56–3·2 μg/g) are in good agreement with those of ERSPAMER for the same species (0·3–2·2 μg/g) as reported by CORREALE (1956). FLOREY and FLOREY (1954) found comparable amounts of 5-HT in optic, cerebral, and stellate ganglia of the European cuttlefish, *Sepia officinalis;* however, they expressed their values (17·5–22·5 μg 5-HT/g) in terms of dry weight of tissue. Reduction of these amounts to 10 percent should give the approximate values per g of fresh tissue. Thus, independent determinations of 5-HT levels in a variety of cephalopods, by a variety of methods, have produced values that are in good agreement.

Some species of *Octopus* (e.g. *Octopus vulgaris*) have long been known to secrete a poisonous substance from the posterior salivary gland capable of paralysing crabs which they have bitten. The posterior salivary glands of *O. vulgaris* and certain other Octopoda contained an active compound eventually identified as 5-HT (see ERSPAMER, 1954*a*, *b*, for references). This is but one of several instances where 5-HT is found as a constituent of a venom apparatus or venom. In two separately extracted glands of *Octopus vulgaris*, from Bermuda, we found values of 68 and 72 μg 5-HT/g of fresh tissue. These values are much lower than those reported by ERSPAMER (1954*a*, *b*) which, for Mediterranean *O. vulgaris*, are given as 420–510 μg/g of fresh tissue. CORREALE (1956) reports somewhat lower values for posterior salivary glands of *O. vulgaris* (172 and 258 μg/g when extraction was 100% complete).

Venus and *Busycon* ganglia have a high level of 5-hydroxytryptophan decarboxylase activity (WELSH and MOORHEAD, 1959*b*). A variety of molluscan tissues from several species have been shown by BLASCHKO and coworkers to contain amine oxidase (e.g. BLASCHKO and HIMMS, 1954; BLASCHKO and HOPE, 1957). Physiological evidence indicates a transmitter role of 5-HT in molluscs (e.g. WELSH, 1953; TWAROG, 1954). Certain drugs known to mimic or block 5-HT (e.g. lysergic acid derivatives) act on molluscan hearts at very low dosage levels (e.g. WELSH and MCCOY, 1957). Levels of 5-HT in molluscan nervous systems may be decreased by injections of reserpine and increased by injections of 5-hydroxytryptophan (WELSH, unpublished). These features of the molluscan nervous system make this invertebrate group suitable for studies on 5-HT metabolism and its role as a transmitter agent.

Phylum Arthropoda

The 5-HT levels were estimated in whole animals, or tissues, from representatives

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of three classes of arthropods. Most nervous tissues had little, if any, 5-HT, while the venom apparatus of two wasps and one scorpion were very rich sources of this amine (Table 1). The results will be discussed by classes.

Class *Crustacea:* Two representatives of the more primitive crustaceans, the water-flea, *Daphnia*, and the brine shrimp, *Artemia*, because of their small size were analysed without dissection. Each extract showed an excitation peak near 300 mμ but the *Artemia* extract contained considerably more 5-HT than did that of *Daphnia*.

Nerve cords of three representative macruran crustaceans were assayed. These were a crayfish, *Orconectes;* the northern American lobster, *Homarus;* and the Bermuda spiny lobster, *Panulirus*. Although there may be low levels of 5-HT in the nervous systems of these animals we believe it best to indicate that the values lie below the stated amounts that could have been confidently measured. No measurable amount of 5-HT was found in the excretory organs ('green glands') of these three macrurans.

Tissues from four brachyuran crustaceans were examined—two species of *Cancer;* the green crab, *Carcinus;* and the West Indian land crab, *Gecarcinus*. The ventral ganglia, which make up the great bulk of the central nervous system of a crab, were assayed and in the case of the two species of *Cancer*, multiple extractions were made. In three extracts of *C. borealis* ganglia, distinct excitation peaks at 300 mμ could be seen, and values were calculated. Since 5-HT was less certainly identified in ganglion extracts of the other species, the values are expressed as less than the amounts that would have been readily measured. An extract from supraoesophageal ganglia ('brain') of *C. borealis* gave a value of 0·08 μg/g tissue (Fig. 2).

FLOREY and FLOREY (1954) reported finding a 5-HT-like substance in ventral ganglia and leg nerves of a variety of Mediterranean decapod crustaceans. Using the rat uterus for bioassay, they obtained a value for *Dromia* leg nerves of 22·5 μg/g dry tissue and for *Dromia* ventral ganglia a value of 18·2 μg/g dry tissue. With the guinea pig ileum, the values for the same extracts were somewhat higher. In terms of wet tissue their values would be roughly one-tenth as great. CORREALE (1956) reports ERSPAMER to have found less than 0·05 μg 5-HT/g of ganglia and leg nerves of *Palinurus vulgaris*, the European spiny lobster.

In view of our difficulty in identifying 5-HT in the central nervous system and leg nerves of most decapod crustaceans, it is of much interest that it is present in the pericardial organs of crabs in relatively large amounts. These structures consist of bundles of axons surrounded by a net-work of fine nerve endings. ALEXANDROWICZ (1953) was the first to describe them in detail and ALEXANDROWICZ and CARLISLE (1953) showed that they contained a substance that was remarkably effective as an excitor agent on the hearts of certain crustaceans. MAYNARD and WELSH (1959) found 5-HT in the pericardial organs, and identified it both by paper chromatography and by the fluorescence method. The values reported for *Cancer borealis* by MAYNARD and WELSH (2·6, 3·2, and 4·0 μg 5-HT/g pericardial organs) are very much larger than those found elsewhere in the nervous system of *C. borealis*. In one determination on pericardial organs of *Carcinus maenas* a value of 2·8 μg/g was found. 5-HT was also identified qualitatively in pericardial organ extracts of *Cancer irroratus*. In addition to 5-HT, pericardial organs contain another cardio-regulator substance with properties of a polypeptide which mimics closely the action of 5-HT on the crustacean heart (MAYNARD and WELSH, 1959).

The excretory organs (green glands) of *Cancer irroratus*, *C. borealis* and *Gecarcinus*, all yielded measurable amounts of 5-HT, in fact, more than was found in any part of the nervous system other than pericardial organs (see General Discussion).

JOHN MUELLER, a student, kindly ran for us a few experiments designed to show

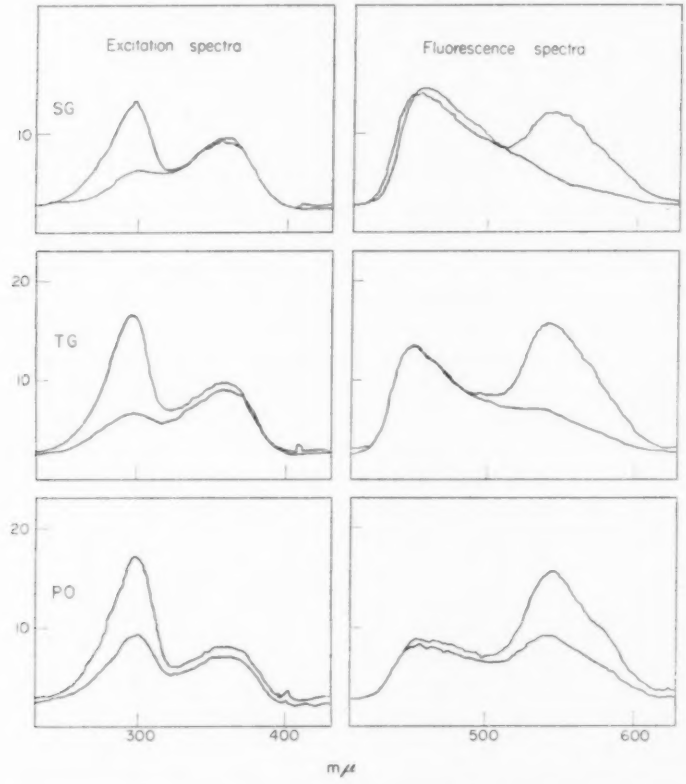

FIG. 2.—Uncorrected excitation and fluorescence spectra of extracts from nerve tissue of the crab, *Cancer borealis*, recorded before (lower curves in each set) and after (upper curves) the addition of 3·0 μg 5-HT to each extract. (SG) Extract from 30 mg supraoesophageal ganglia ('brain') per ml 3 N-HCl. (TG) Extract from 630 mg thoracic ganglia per ml 3 N-HCl. (PO) Extract from 10 mg pericardial organ tissue per ml 3 N-HCl. Note that the additional 5-HT elevates the excitation peak near 300 mμ and the fluorescence peak near 540 mμ while having little or no effect on the other peaks which were also present in the reagent blank. Records have been retraced to facilitate reproduction.

if 5-hydroxytryptophan decarboxylase is present in homogenates of ventral ganglia of *Cancer borealis*. In four experiments, to amounts of tissue ranging from 75 to 120 mg, 200 μg of 5-hydroxytryptophan (5-HTP) were added, and the mixture was shaken for 3 hr at 18–22°. Controls were run without added 5-HTP, some with an amine oxidase inhibitor (iproniazid) and some without. At the end of 3 hr, extracts were made and examined with the spectrofluorometer. No control gave a detectable amount of 5-HT, while with added 5-HTP the values found were 2·4, 2·7, 2·9, and 5·6 μg/g of original tissue. Thus 5-HTP decarboxylase is present in small amount in

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the ventral ganglia of *C. borealis*. Compared with some molluscs, however, its level is low (cf. WELSH and MOORHEAD, 1959*b*). We shall return to the possible significance of this in the General Discussion.

Class *Arachnoidea:* The so-called horse-shoe crabs have been in continuous existence, with relatively little change in physical appearance, for a period estimated to be some 500,000,000 years. It is of much interest, therefore, to find 5-HT readily demonstrable in the nervous system of the American representative of this primitive group, *Limulus polyphemus*. The values are low, but a single nerve cord or the leg nerves of one specimen provided enough 5-HT to be measured readily with the spectrofluorometer. The heart with its ganglion attached, as well as the intestine, contained 5-HT, but more was found in the coxal glands (excretory organs) than in any other tissue that was studied (see General Discussion).

ADAM and WEISS (1956, 1958) found an extremely large amount of 5-HT in the venom of the scorpion, *Leiurus quinquestriatus*. They estimated that a gram of dry venom from this species contains 2000 to 4000 μg 5-HT. A much smaller amount of 5-HT (30–40 μg/g dry venom) was found in another scorpion, *Buthotus minax* (ADAM and WEISS, 1959). We obtained three species of scorpions from Arizona, two of which were identified as *Hadrurus arizonensis* and *Vejovis spinigerus*, while the third was believed to be another species of *Vejovis*. The stinging segment of two specimens of each species was assayed for 5-HT. To our surprise there was no detectable 5-HT in extracts from *Hadrurus* and *Vejovis spinigerus* while extracts of two supposed *Vejovis sp.* yielded 50 μg and 138 μg per g of terminal segments consisting largely of venom apparatus. The onset of the winter season prevented securing more specimens of '*Vejovis sp.*', and these preliminary results are recorded only to show that some scorpions may have no 5-HT in their venom apparatus. Chromatography of extracts of the venom apparatus of *Hadrurus arizonensis* and *Vejovis spinigerus* failed to show the presence of histamine, which is abundant in bee venom and that of the wasp *Vespa vulgaris* (JACQUES and SCHACHTER, 1954).

The presence of a large amount of interfering substance in extracts of head and thorax, and of abdominal segments of the scorpions available to us, obscured possible 5-HT activation and fluorescence peaks. It would probably be necessary to dissect nervous tissue from large scorpions in order to determine whether or not 5-HT is present there.

Class *Insecta:* An extract of ten ventral nerve cords of the large tropical cockroach, *Blaberus gigantea*, failed to give either excitation or fluorescence peaks characteristic for 5-HT. An extract from heads and the first thoracic segment of the same species, however, gave a distinct excitation peak. Small amounts of 5-HT were found in extracts of whole larvae of the mealworm, *Tenebrio*, of the head and thorax of the mud-dauber, *Sceliphron*, and of the head of the honey-bee. It is not possible to say how much of this derived from nervous tissue. CORREALE (1956) quotes ERSPAMER as estimating the 5-HT content of cerebral ganglia of *Locusta migratoria* to be less than 0·05 μg/g tissue.

JACQUES and SCHACHTER (1954) found about 300 μg 5-HT/g dry venom of the European wasp, *Vespa vulgaris*, while ERSPAMER (1954*a*) reported finding 0·7–0·8 μg 5-HT/venom apparatus (2·5 mg) of the wasp, *Polistes gallica*. Extracts of whole abdomens of the mud-dauber wasp, *Sceliphron*, and an unidentified species of *Polistes* had a very high content of 5-HT. If it be assumed that all of the 5-HT is in the venom

apparatus, *Sceliphron* has 0·7 μg/apparatus and *Polistes sp.* has 1·0 μg/apparatus. The values correspond closely with that for *Polistes gallica* as determined by ERSPAMER (1954*a*).

ERSPAMER (1954*a*) reported that he and PAVAN could not identify 5-HT in extracts of venom apparatus of the honeybee, *Apis mellifera*. NEUMANN and HABERMANN (1956) also failed to find 5-HT in bee venom. With the method of extraction and assay used by us, appreciable amounts of 5-HT were first demonstrated in extracts from tips of the abdomen and then in an extract from isolated stinging apparatus. For the venom apparatus a value of 21 μg/g was obtained or about 0·03 μg/apparatus (1·5 mg). In order to make the identification more certain, 63 mg of venom apparatus from *Apis* were treated in ethanol and chromatographed. The very sensitive spray reagent (*p*-dimethylaminocinnamaldehyde) recommended for visualizing indole derivatives (HARLEY-MASON and ARCHER, 1958) was used. The bee venom apparatus extract gave a spot whose colour and R_F value were indistinguishable from those of the standard 5-HT. Using paper chromatography, we also showed the presence of a very large amount of histamine in the ethanol extract from bee venom apparatus, thereby confirming earlier observations by other workers (see NEUMANN and HABERMANN, 1956, for references).

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While insect nervous systems, or parts thereof, probably contain 5-HT, its level is low as in the other arthropods that have been examined. Venoms of some of the Hymenoptera, however, are a rich source of 5-HT.

Phylum Echinodermata

As the result of their reversion to radial symmetry, the echinoderms lost such centralization of the nervous system as their bilaterally symmetrical ancestors may have possessed. The diffuse nature of the nerve plexuses in the echinoderms makes it well-nigh impossible to obtain samples of nerve tissue sufficient for extraction and 5-HT assay. Our effort was directed, therefore, to determining whether or not 5-HT occurs in representative North Atlantic echinoderms. Examples from three of the five existing classes were examined. In three cases (*Asterias*, a starfish; *Strongylocentrotus*, a sea urchin; *Synapta*, a sea cucumber) extracts from whole animals or from animals with gonads removed were made. Large weights of tissue were treated, and the 5-HT was partitioned into the usual small volume of HCl. With this procedure, 5-HT excitation and fluorescence peaks were obvious and values could be calculated although they are low (Table 1). In the sea cucumber, *Thyone*, the general region of the nerve ring was removed and assayed. The values were not significantly larger than those obtained for the other species.

While 5-HT is present in the echinoderms, it is not possible from our results to say to what extent it is concentrated in the nervous system.

Phylum Chordata

Molgula, an ascidian, was selected as a representative of the protochordates. As in the echinoderms, the nervous system is largely diffuse. Different parts of *Molgula* were analysed. An interfering substance was present in all extracts and no certain identification of 5-HT could be made. However, we were furnished with about 350 ganglia in acetone from the West Coast ascidian, *Chelyosoma productura*. After evaporating the acetone we obtained a total of 0·3 μg 5-HT from the residue.

Since some of the acetone may have leaked in transit, this value only indicates the presence of 5-HT in the ganglionic complex of *Chelyosoma*.

GENERAL DISCUSSION

5-HT *in Nervous Tissues*

5-HT has been shown to be present in primitive nervous systems—possibly the most primitive existing at the present time. It certainly occurs in annelid nervous systems. The evidence indicates its presence in nervous systems of flatworms and ribbon worms, but further work is necessary to prove its presence in the coelenterate nervous system, although its occurrence in coelenterates has been amply demonstrated.

More 5-HT is found per unit weight of tissue in ganglia of the bivalve molluscs than in any other nerve tissue thus far examined, either vertebrate or invertebrate. The mean value of 40 μg 5-HT/g of *Venus* ganglia, which is approximated only by ganglia of certain other pelecypod molluscs, is 20–200 times the levels found in parts of the mammalian CNS that are considered to be relatively rich in 5-HT (cf. CRAWFORD, 1958). The nervous system of the most highly evolved and active molluscs, the cephalopods, have the least 5-HT found in any mollusc examined by us. One might conclude from this that the squid, octopus, and their relatives use their 5-HT more efficiently than do the clams, oysters, and their relatives. It has been shown that formation of 5-HT from 5-hydroxytryptophan (5-HTP) by an homogenate of *Venus* ganglia takes place at a high rate (WELSH and MOORHEAD, 1959*b*). It would be of interest to know the corresponding rate for some cephalopod ganglia.

The presence of lower amounts of 5-HT in molluscan nerve than in ganglia suggests that it is concentrated in cell bodies or nerve terminals. From our knowledge of the concentration of other neurohormones, including neurosecretory substances, in presynaptic terminals (WELSH, 1955), it seems probable that more 5-HT will be found in the region of ganglionic neuropiles of molluscs than elsewhere in their nervous system.

Many of our attempts to demonstrate 5-HT in ganglia and leg nerves of arthropods were negative. When it was found, the amounts were low compared with annelids and molluscs. Pericardial organs were an exception to be discussed later. Among the crabs, lobsters, and a crayfish, only in *Cancer borealis* could we detect an activation peak in extracts of ganglia, and then only when 1–2 g of tissue were extracted. Crustacean pericardial organs, which are nerve plexuses, were found, on the other hand, to contain amounts of 5-HT ranging from 2·5 to 4 μg/g. We were able to measure the amount of 5-HT extracted from as little as 27 mg of pericardial organ tissue of the green crab, *Carcinus maenas*. Since pericardial organs consist of axons of ordinary neurons surrounded by a network of fine terminals of neurosecretory cells, it would be of interest to determine the exact location of the stored 5-HT. When assayed on a crustacean heart, extracts of pericardial organs appear to have an extremely high 5-HT content. However, this has been shown to be due largely to the presence of another substance, probably a polypeptide, that closely mimics the action of 5-HT on the crustacean heart (MAYNARD and WELSH, 1959). It is of much interest to find two such unrelated substances as an indole amine and a polypeptide both deriving from the same nerve plexus and having similar actions on a given target organ, in this case the heart.

Attention was directed earlier to the great antiquity of the horse-shoe crab,

Limulus, which, of course, is not a true crab, and represents a small group of animals with no close relatives among existing forms. 5-HT was found in extracts of *Limulus* nerve cord and leg nerves. It is tempting to suggest that in the evolution of the arthropods, as well as the molluscs, less 5-HT is required in the more highly evolved groups and less 5-HT is stored at any given time. Preliminary studies have shown that homogenates of ganglia of *Cancer borealis* decarboxylate 5-HTP at a much lower rate than do homogenates of molluscan ganglia.

We extracted cleaned nervous tissue of only one insect, the giant cockroach, *Blaberus gigantea*. Ten ventral nerve cords weighing 160 mg were analysed; the extract, examined in the spectrofluorometer, failed to show distinct excitation and fluorescence peaks. However, when heads and first thoracic segments of *Blaberus* were used the extracts gave distinct peaks that quite certainly represented 5-HT. Heads of a wasp, *Sceliphron*, and the honey bee, *Apis mellifera*, likewise yielded significant amounts of 5-HT. It is possible that there are structures in the head region of insects such as the corpora cardiaca or allata that have a high level of 5-HT, but the occurrence of 5-HT in the 'brain' of one of the crabs suggests that isolated supraoesophageal ganglia of insects should be examined. That some insects have the necessary enzyme systems for synthesizing 5-HT may be deduced from the presence of large amounts of this indole amine in some venoms.

Whether the 5-HT that is readily demonstrable in extracts of echinoderms is of nervous or extra-nervous origin will be determined only when isolated nervous tissue is examined. *Chelyosoma*, which may be considered to represent a group that is intermediate between echinoderms and vertebrates has 5-HT in its nervous system.

CORREALE (1956) has demonstrated the presence of 5-HT in brains of representatives of the five classes of vertebrates. It seems probable that this indole alkyl amine is present in nervous systems of most, if not all, animal phyla.

That 5-HT has multiple physiological roles seems clear (cf. PAGE, 1958). That one of these is as a neurohumor, or transmitter agent, is slowly being established. Some of the earliest evidence for this came from work on invertebrates. The early observation of the action of enteramine on molluscan hearts (ERSPAMER and GHIRETTI, 1951) was followed by observations on the comparable effects of applied 5-HT and cardiac nerve stimulation on the *Venus* heart (WELSH, 1953, 1957). An inhibitor action of 5-HT on molluscan body muscle was first observed by TWAROG (1954). Unpublished studies of our own on the effects of injected reserpine and 5-HTP on molluscan behaviour add further strength to the view that 5-HT is an important agent of the molluscan nervous systems.

Crustacean hearts respond to very low concentrations of 5-HT (FLOREY and FLOREY, 1954), and some crustacean sense organs are excited by low doses of applied 5-HT (FLOREY, 1954). This, and other evidence, points to a humoral role of this amine in the Crustacea.

5-HT has an action opposing that of acetylcholine in some invertebrates (see WELSH, 1953; TWAROG, 1954; HILL, 1958), as in the vertebrates, at some locations. It is of interest that acetylcholine is widely distributed in invertebrate nervous systems (PROSSER, 1946). Whatever parts acetylcholine and serotonin may play in the functioning of the hypothalamus and elsewhere in the central nervous systems of man and other mammals, it is certain that these will not prove to be attributes only of the so-called higher animals.

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5-HT *in non-nervous tissues*

The 5-HT found in such structures as the *Venus* heart or mantle (WELSH and MOORHEAD, 1959*a*) and *Limulus* intestine (this paper) may well be of nervous origin and derive from nerve endings. Certain tissues and organs of invertebrates, however, have a high content of 5-HT of extra-nervous origin. ERSPAMER and co-workers (see ERSPAMER, 1954*a*, *b*, *c*), discovered many rich natural sources of enteramine (later identified as 5-HT) such as the hypobranchial gland of *Murex trunculus* (80–290 μg/g), the posterior salivary gland of *Octopus vulgaris* (420–510 μg/g , and the skin of amphibia, e.g. the toad, *Bombinator pachypus* (700–1000 μg/g). Other rich sources of 5-HT in the invertebrates are venoms and venom apparatus of some scorpions (ADAMS and WEISS, 1956, 1958, 1959; and this paper) and wasps (JACQUES and SCHACHTER, 1954; ERSPAMER, 1954*a*; and this paper). In most of ERSPAMER's studies, chromaffin cells have been found in the venom apparatus showing histochemical reactions similar to those seen in the enterochromaffin cells of the vertebrate intestine (see ERSPAMER, 1954*a*, *b*). ADAM and WEISS (1959) characterized such cells in the epithelium of the venom apparatus of the scorpion, *Leiurus quinquestriatus*. That 5-HT may be a constituent of coelenterate venoms has already been discussed (p. 167).

5-HT is relatively non-toxic and seems not to contribute directly to the toxicity of the venoms in which it occurs. For example, ADAMS and WEISS (1956) removed most of the 5-HT from the venom of *L. quinquestriatus* by treatment with 95% acetone without affecting the toxicity of the venom. Through its ability to produce pain (ARMSTRONG, DRY, KEELE and MARKHAM, 1953), 5-HT may have survival value (ADAMS and WEISS, 1959). It relaxes molluscan body muscle and may serve to relax the molluscan prey of the carnivorous gastropods (e.g. species of *Murex* and *Thais*) that have hypobranchial glands containing 5-HT (WELSH, unpublished). However, its most important role in venoms could be facilitation of the absorption and distribution of the true toxic components through its own action on permeability and circulation (see PAGE, 1958), as well as the actions of the histamine that it releases.

In the course of this study, excretory organs of a number of molluscs and arthropods were found to have relatively high levels of 5-HT. Such organs are not well-innervated, and their 5-HT almost certainly does not derive from intrinsic nerve endings. In *Limulus*, for example, more 5-HT was found in coxal glands than in any other tissue examined, including the nerve cord (Table 1). In the crabs, *Cancer irroratus* and *Gecarcinus lateralis*, the 'green glands', which are their excretory organs, were the only structures to yield sufficient 5-HT for estimation by the fluorescence method. We had earlier reported a relatively high level of 5-HT in the kidney of *Busycon* (WELSH and MOORHEAD, 1959*a*), and comparable amounts were found (Table 1) in the kidneys of two other marine gastropods, *Melongena* and *Fasciolaria*. In one determination on the kidneys of the fresh-water clam, *Anodonta*, a value of 1·3 μg/g was obtained. We were unable to detect 5-HT in the green glands of the crayfish, *Orconectes*, or the lobster, *Homarus*.

When homogenates of *Busycon* kidney were incubated with added 5-HTP, neither was there any new 5-HT formed nor was the original tissue 5-HT destroyed; this result suggests the absence of both 5-HTP decarboxylase and monoamine oxidase from this organ (WELSH and MOORHEAD, 1959*b*). On the other hand, similar preliminary experiments with coxal glands of *Limulus* and green glands of *Cancer*

borealis gave evidence for the presence of both of these enzymes. It is possible that some invertebrate excretory organs are removing 5-HT from the blood and concentrating it for excretion or for some intrinsic role. Other excretory organs may be able to synthesize their own 5-HT.

There seems yet to be no certain answer to the question of a direct regulatory control of 5-HT on mammalian kidney function as first postulated by ERSPAMER (see PAGE, 1958, for discussion and references). Perhaps work with invertebrate kidneys will provide useful clues to the possible role of 5-HT in water and electrolyte regulation. It is entirely conceivable that regulation of ion flux, whether in kidney, smooth muscle, sense organ, the central nervous system, or elsewhere, will prove to be the basic mechanism of action of 5-hydroxytryptamine.

SUMMARY

The fluorescence method has been used to estimate the 5-hydroxytryptamine (5-HT) content of nervous tissues from a variety of invertebrates. Certain other tissues and organs have been assayed for comparison. Representative annelids and molluscs were found to have relatively large amounts of 5-HT in their nervous systems, while arthropods have very little. Some evidence was found to suggest that the more primitive and less specialized invertebrates have more 5-HT in their nervous systems than do the more specialized forms. 5-HT was found in coelenterates and flatworms, the most ancient animals with a nervous system, but isolated nervous tissue was not studied. The venom apparatus from some species was found to have a high content of 5-HT in confirmation of observations by other workers.

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H. G. KNAUFF and F. BÖCK: Über die freien Gehirnaminosäuren und das Äthanolamin der normalen Ratte, sowie über das Verhalten dieser Stoffe nach experimenteller Insulinhypoglykämie.

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M. NIEMI and E. MERENMIES: Cytochemical localization of the oxidative enzyme systems in the retina—I. Diaphorases and dehydrogenases.

M. NIEMI and E. MERENMIES: Cytochemical localization of the oxidative enzyme systems in the retina—II. Cytochrome oxidase.

E. L. BENNETT, M. R. ROSENZWEIG, D. KRECH, A. OHLANDER and H. MORIMOTO: Cholinesterase activity and protein content of rat brain.

R. P. KAMRIN and ANNE A. KAMRIN: The effects of pyrindoxine antagonists and other convulsive agents on amino acid concentrations of the mouse brain.

R. KUNTZMAN, P. A. SHORE, D. BOGDANSKI and B. B. BRODIE: Microanalytical procedures for fluorometric assay of brain DOPA-5HTP decarboxylase, norepinephrine and serotonin, and a detailed mapping of decarboxylase activity in brain.

L. D. MACLEOD: The distribution of the arylamino group in the brain of the albino rat following administration of *p*-aminobenzoic acid

H. MCILWAIN: Apparatus for applying electrical pulses to isolated cerebral tissues during metabolic studies.

K. F. GEY and A. PLETSCHER: Activity of monoamine oxidase in relation to the 5-hydroxytryptamine and norepinephrine content of the rat brain.

J. KŘIVÁNEK: Some metabolic changes accompanying Leão's spreading cortical depression in the rat.

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ÜBER DIE FREIEN GEHIRNAMINOSÄUREN UND DAS ÄTHANOLAMIN DER NORMALEN RATTE, SOWIE ÜBER DAS VERHALTEN DIESER STOFFE NACH EXPERIMENTELLER INSULINHYPOGLYKÄMIE

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BEI vorausgehenden Untersuchungen über die freien Aminosäuren des Liquor cerebrospinalis (KNAUFF 1958; KNAUFF, MIALKOWSKY und ZICKGRAF, 1959) konnten wir erstmals freies Äthanolamin in beachtlicher Konzentration nachweisen (KNAUFF und ZICKGRAF, 1958). Im Blutserum ist es nur in Spuren vorhanden (WALKER, 1952; KNAUFF, SELMAIR und REITLINGER, 1960), während die meisten freien Aminosäuren dort in höherer Konzentration vorliegen. Das Liquoräthanolamin scheint daher hauptsächlich aus der Hirnsubstanz und nicht direkt aus dem Serum zu stammen. Das Gehirn enthält als kephalinreichstes Organ des Körpers sehr viel freies (TALLAN, MOORE und STEIN, 1954) und gebundenes (KLENK und BÖHM, 1951; KLENK, 1951) Äthanolamin, letzteres vor allem in der Fraktion der Acetalphospholipide (KLENK, DEBUCH und DAUN, 1953; STAMMLER, STAMMLER und DEBUCH, 1954). Da die Bluthirnschranke für diese Stoffe unpassierbar ist (SLOANE-STANLEY, 1952) müssen sie von der Gehirnzelle aus niedermolekularen Grundbausteinen auf und wieder zu diesen abgebaut werden (SLOANE-STANLEY, 1951, 1952, 1953). Dabei gelangen sie in die Extrazellulärflüssigkeit und damit in den mit dieser kommunizierenden Liquor. In den Liquorraum eingebrachtes radioaktives Phosphat wird umgekehrt von den Gehirnzellen aufgenommen und in Phosphatide eingebaut (STRICKLAND, 1952).

Der Phosphatidumsatz des Gehirns scheint zwar unter physiologischen Verhältnissen grösser zu sein (DAWSON und RICHTER, 1950), als man früher (CHAIKOFF, 1942) annahm, und die Kephaline werden dabei von allen Gehirnphosphatiden am schnellsten umgebaut (TYRRELL, 1950; SLOANE-STANLEY, 1952; DAWSON, 1954). Trotzdem sind sie vorwiegend als Baustoffe aufzufassen. Ihre Stoffwechselrate ist geringer als die der labileren Energiestoffe. Jeder plötzliche Phosphatidabbau, der an sich fermentmässig möglich ist (TYRRELL, 1950; SLOANE-STANLEY, 1951, 1952), sollte daher in Gehirn und Liquor mit einer messbaren Vermehrung von Phosphatidspaltstücken wie Äthanolamin, Serin, Cholin und Inosit einhergehen, vorausgesetzt, dass die Phosphatidspaltung schneller erfolgt, als der Endabbau der freiwerdenden Bruchstücke. Deren Bestimmung würde dann indirekt den Schluss auf einen im Gehirn vorsichgehenden Phosphatidabbau erlauben.

Eine Möglichkeit dies zu prüfen bietet das experimentelle *Insulinkoma*. Dabei wird nämlich ein ungewöhnlich starker Abbau der Hirnlipoide, insbesondere der Phosphatide beobachtet (RANDALL, 1940; MCGHEE *et al.*, 1951), der schon wenige Stunden nach einer einmaligen grossen Insulinapplikation 10 Prozent der gesamten Hirnphosphatide betragen kann. Dieser Effekt soll auch dann eintreten, wenn durch

gleichzeitige laufende Glukosegaben ein Abfall des Blutzuckerspiegels, hypoglykämische Krämpfe und ein Insulinkoma verhindert werden (McGHEE *et al.*, 1951). Ob dann Störungen des intermediären Zuckerstoffwechsels den Abbau der Phospholipoide und ihre Mitverwendung als energielieferndes Substrat erfordern, ist ungeklärt. Wiederholte Insulinapplikation in grösseren Dosen führt schliesslich zu Zelluntergang und histologischen Veränderungen (GOZZANO, 1929; BODECHTEL, 1933; TANI 1935; WEIL *et al.*, 1938).

Seit einigen Jahren wissen wir ferner, dass im Insulinkoma auch gewisse freie Gehirnaminosäuren stark abfallen (DAWSON, 1949, 1950; CRAVIOTO *et al.*, 1951). Sie dürften als Oxydationssubstrat an Stelle der Glukose in den Stoffwechsel eingeschleust werden (DAWSON, 1950). Wahrscheinlich ist dies ausser bei Aminosäuren und Phosphatiden, auch bei Nucleinsäuren und anderen Strukturbestandteilen des Gehirns möglich (GEIGER *et al.*, 1952; ABOOD und GEIGER, 1955; ALLWEIS und MAGNES, 1958).

Auch bei der mit gesteigertem Stoffwechsel einhergehenden direkten elektrischen Reizung des Gehirns, wurde ein starker Verbrauch an freien Aminosäuren beobachtet (GEIGER, DOBKIN und MAGNES, 1953). Wir haben daher unsere Untersuchungen auch auf die freien Aminosäuren ausgedehnt. Es sollte erstens geklärt werden, ob es bei dem im Insulinkoma anzunehmenden Phosphatidabbau zum Freiwerden und zur Anhäufung niedermolekularer Phosphatid-Bausteine, wie Äthanolamin, Serin und Cholin kommt und ob zweitens gleichzeitig andere, zur Energielieferung gebrauchte Aminosäuren verschwinden. Wir erwarteten dabei ein gegensätzliches Verhalten dieser beiden Stoffgruppen.

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METHODIK

Material

Als Versuchstiere dienten 80 Albinoratten im Gewicht von 160–200 g. 46 Ratten wurden für Versuche zur Standardisierung der Methodik, sowie zur Ermittlung der günstigsten Insulindosis und Schockdauer verwendet. Diese Serien sind für die statistische Auswertung zu klein und bleiben daher unberücksichtigt. Die mitgeteilten Ergebnisse beziehen sich nur auf die zuletzt in den Versuch genommene Rattenserie von 34 Tieren. 15 dienten als Kontrollen, 19 bekamen Altinsulin ('Horm') in einer Dosis von 0·1 IE/g Körpergewicht (14–20 IE/Ratte) subcutan. Schon nach 30 Minuten sträubten die Tiere ihr Fell, wurden unruhig und schreckhaft. Später wurden sie ruhiger und nach 2 Stunden zunehmend apathisch. Nach etwa 4 Stunden traten die ersten tonisch-klonischen Krämpfe auf. Nach 5 Stunden wurden sie in tiefer Somnolenz durch einen kräftigen Scherenschlag dekapitiert und der Kopf zur Unterbrechung weiterer Stoffwechselvorgänge, sofort in flüssige Luft getaucht. Dies ist notwendig, da sich sonst infolge der im Gehirn vorhandenen Proteinaseaktivität der Aminosäuregehalt schnell ändert (SCHURR *et al.*, 1950; ANSELL und RICHTER, 1954*a*). Die in gleicher Weise getöteten Normalratten wurden vorher etwa 1 Minute lang ganz leicht mit Äther narkotisiert. Eine tiefere Narkose wurde mit Absicht vermieden, da diese ebenfalls die Konzentration der freien Gehirnaminosäuren verändern kann (RICHTER und DAWSON, 1948; DAWSON, 1951)

Gewinnung und Aufarbeitung der Extrakte

Die fest gefrorenen Köpfe wurden mit dem Skalpell halbiert. Das Gehirn wurde vorsichtig herausgelöst, gewogen (Gehirngewichte 1·6–1·9 g) und dann 3 Minuten mit 10 ml einer vorgekühlten Salzsäure-Acetonlösung (1 Teil 0·1 N-HCl auf 4 Teile Aceton) im Homogenisator nach Potter homogenisiert. Das Homogenat wurde 15 Minuten bei 12000 U/Min. zentrifugiert und die überstehende klare Lösung durch vorsichtiges Dekantieren vom Eiweiss- und Salzniederschlag getrennt. Der Rückstand wurde mit salzsaurem Aceton ausgewaschen und erneut abzentrifugiert. Dieses Verfahren lieferte die grösste Aminosäureausbeute. Bei Extraktion mit weniger HCl, oder mit Aceton oder Alkohol allein, war die Ausbeute geringer. Beide Zentrifugate wurden vereinigt, zur

Trockene eingeengt und dann mit 8 ml dest. Wasser aufgenommen. Diese Lösung wurde zur Entfernung der Lipoide 2 mal mit Äther extrahiert und nach CONSDEN, GORDON und MARTIN (1947) elektrolytisch entsalzt. Dazu fand die früher beschriebene (KNAUFF, MIALKOWSKY und ZICKGRAF, 1959) modifizierte Astrup-Apparatur Verwendung, die eine intensive Kühlung der zu entsalzenden Lösung gestattet. Die Entsalzungszeit betrug einheitlich 20 Min., die Temperatur stieg nicht über 10°. Bei Einhaltung dieser Bedingungen können Aminosäureverluste weitgehend vermieden werden. Sie liegen für die meisten Aminosäuren unter 3%. Lediglich bei Arginin wurde ein höherer Verlust von etwa 15% bestimmt. Vorversuche mit eingewogenen Aminosäuren in Ringerlösung ergaben bei genauer Standardisierung des Verfahrens einheitliche Verlustquoten von Versuch zu Versuch. Unsere Ergebnisse werden daher durch die elektrolytische Entsalzung der Lösungen nicht wesentlich verfälscht. Die entsalzte Lösung wurde filtriert. Das Filter wurde mehrmals mit dest. Wasser nachgewaschen und dann mit Ninhydrin gefärbt, um Verluste beim Filtrieren auszuschliessen. Färbte sich das Filter noch schwach an, wurde der ganze Versuch verworfen. Filtrat und Waschwasser wurden vereinigt und im Vakuum vorsichtig zur Trockene eingeengt. Der Rückstand wurde mit 0·15 ml 0·1 N-HCl pro Gramm ursprüngliches Gehirngewicht aufgenommen und zur Chromatographie verwendet.

Qualitative Bestimmung

Die qualitative Bestimmung nach einem früher beschriebenen kombinierten hochspannungselektrophoretisch-papierchromatographischen Verfahren (KNAUFF, MIALKOWSKY und ZICKGRAF, 1959) diente vorwiegend der Erfassung der nur in Spuren vorhandenen Stoffe. Dabei werden die punktförmig aufgetragenen Substanzen zunächst in erster Dimension elektrophoretisch und dann senkrecht dazu auf dem gleichen Bogen papierchromatographisch getrennt. Auftragsmenge einheitlich 0·02 ml, entsprechend 133·3 mg Hirngewebe. Zur Hochspannugselektrophorese verwendeten wir eine modifizierte Apparatur nach WERNER und WESTPHAL (1955). Puffer: Ameisensäure-Eisessig-Wasser 1:3:16 (Ph 1·9); Klemmspannung: 3000 V (50 V/cm); Stromstärke: 25 mA; Elektrophoresedauer: 90 Min; Papier: Schleicher und Schüll 2043b (Format 35 × 60 cm). Nach der Elektrophorese wurden die Bögen 2x je 16 Stunden in Butanol-Eisessig-Wasser (4:1:5) aufsteigend chromatographiert und dann mit Ninhydrin nach LEVY und CHUNG (1953) gefärbt. Für weitere methodische Einzelheiten verweisen wir auf frühere Arbeiten (KNAUFF und ZICKGRAF, 1958; KNAUFF, MIALKOWSKY und ZICKGRAF, 1959).

Zum besseren Nachweis der sich mit Ninhydrin schlechter anfärbenden, oder nur in Spuren vorhandenen Aminosäuren dienten Spezialfärbungen. Wir fertigten zu diesem Zweck getrennte, eindimensionale Elektropherogramme an und trugen hier jeweils grössere Substanzmengen (0·08 ml, ensprechend 533 mg Hirngewebe) auf. Verwendet wurde die Isatinfärbung nach ACHER *et al.* (1950) (Prolin: blau, Lysin: rosa, Phenylalanin: grün, Tryptophan: rostbraun), die Pauly' sche Diazoreaktion (Histidin: kirschrot, Tyrosin: rot) und die für Methionin spezifische Färbung mit Platintetrachlorid und Natriumjodid (vgl. TURBA, 1954). Cholin konnte mit der Färbung nach LEVINE und CHARGAFF (1951) in der Modifikation von BEVAN *et al.* (1951) nachgewiesen werden. Es färbte sich dabei leuchtend blau und war auf zweidimensionalen Elektrophero-Chromatogrammen durch seine Position von anderen, ebenfalls diese Reaktion gebenden Stoffen unterscheidbar.

Quantitative Bestimmung

0·01 ml derselben Lösung, die zur Anfertigung der kombinierten Elektrophero-Chromatogramme benutzt wurde (entsprechend dem Extrakt aus 66·76 mg Gehirn), trugen wir strichförmig auf 4 cm breite, 40 cm lange, parallele Streifen auf, die durch 1 cm breite Einschnitte voneinander getrennt waren und nur am unteren und oberen Rand miteinander zusammenhingen. Der ganze Filterpapierbogen (Schleicher und Schüll 2043b), war 19 cm breit und in 4 solche Streifen unterteilt. Auf einen Streifen kam der Gehirnextrakt, auf die benachbarten Streifen wurden genau bekannte, ähnlich zusammengesetzte Testaminosäuregemische in verschiedener Konzentration aufgetragen. Mit jedem Hirnextrakt wurden 3 Filterpapierbogen beschickt, die in 3 verschiedenen Laufmitteln (Butanol-Eisessig-Wasser, 4:1:5; Methyläthylketon-Pyridin-Wasser, 7:1·5:1·5; *Iso*propanol-Eisessig-Wasser 7:1·5:1·5), aufsteigend chromatographiert wurden. Nach 24 Stunden wurden die Streifen getrocknet, anschliessend für weitere 24 Stunden im gleichen Laufmittel chromatographiert und dann mit Ninhydrin nach BODE (1955) gefärbt. Sodann wurden die einzelnen Streifen, auf denen meistens 10–12 deutlich getrennte Banden sichtbar waren, vom Bogen getrennt, transparent gemacht

und mit einem automatisch schreibenden Extinktionsmessgerät (Elphorintegraph, Bender and Hobein), bei der Wellenlänge $\lambda = 578$ mμ, dem Extinktionsmaximum der entstehenden Ninhydrinfärbung, vermessen. Die Extinktionskurven wurden in üblicher Weise ausplanimetriert und die erhaltenen Werte auf die Werte der gleichzeitig auf demselben Bogen mitchromatographierten Testlösungen bekannter Konzentration bezogen. Fehler durch ungleichmässige Färbung von Bogen zu Bogen, durch wechselnde Retention im Papier etc. werden so weitgehend ausgeschaltet. Die Fehlergrenze für papierchromatographische Aminosäurebestimmungen liegt bei diesem Vorgehen bei $\pm 3\%$ (GRASSMANN, HANNIG und PLÖCKL, 1955; KNAUFF, DIETERLE und ZICKGRAF, 1959). Die in jedem Falle durchgeführte Parallelchromatographie in 3 verschiedenen Laufmitteln gestattete, die meisten Aminosäuren auf wenigstens einem Streifen von allen anderen abzutrennen und getrennt zu bestimmen. Nur Asparaginsäure, *Iso*leucin-Leucin, Lysin-Ornithin, Serin, Threonin und Valin bildeten auf keinem Streifen eine selbstständige Bande. Asparaginsäure, Serin, Threonin und Valin waren jedoch mit einer Aminosäure kombiniert, deren Menge auf einem zweiten Streifen einzeln bestimmt werden konnte. Ihr Extinktionswert wurde errechnet und von dem der gemeinsamen Bande abgezogen. Die Differenz konnte dann auf die sonst nicht trennbare Aminosäure bezogen werden. *Iso*leucin-Leucin und Lysin-Ornithin wurden nur gemeinsam bestimmt. Die fertig ausgewerteten Streifen wurden später entfettet und zur Haltbarmachung mit Kupfer (0·4 ml Acidum nitricum + 2 ml Cuprum nitricum + 200 ml Aceton) besprüht. Methodische Einzelheiten wurden bereits an anderer Stelle (KNAUFF, DIETERLE und ZICKGRAF, 1959) veröffentlicht.

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ERGEBNISSE

A. Normalratten

Abb. 1 zeigt ein mit Ninhydrin gefärbtes Elektrophero-Chromatogramm vom Gehirnextrakt einer Kontrollratte.

Man erkennt 18–20 durch verschiedene Aminosäuren und Amine hervorgerufene Flecken. Im Orginal-Chromatogramm heben sie sich wesentlich deutlicher voneinander ab, da die Färbung nach Levy und Chung sehr differente und für die einzelnen Aminosäuren charakteristische Farbnuancen liefert. Es sind dort oft noch einige schwächere, auf der Abb. 1 nicht sichtbare Flecken erkennbar. Folgende Stoffe wurden regelmässig nachgewiesen : Äthanolamin, Alanin, γ-Aminobuttersäure, Arginin, Asparaginsäure, Cystin, Glutamin, Glutaminsäure, Histidin, *Iso*leucin-Leucin, Lysin, Methionin, Ornithin, Phenylalanin, Phosphoäthanolamin, Prolin, Serin, Taurin, Threonin, Tryptophan, Tyrosin und Valin. Dazu kamen manchmal einige undefinierte Substanzen, die sich ebenfalls mit Ninhydrin färbten, wahrscheinlich Peptide. Die stärksten Flecke wurden durch Glutaminsäure, Glutamin, γ-Aminobuttersäure, Äthanolamin, Phosphoäthanolamin und Taurin verursacht. Der Nachweis von Arginin, Methionin und Prolin gelang auf besonderen, mit Spezialfärbungen behandelten, eindimensionalen Chromatogrammen. Ferner wurde stets Cholin qualitativ nachgewiesen. Auch dazu bedienten wir uns einer Spezialfärbung auf eigenem Chromatogramm.

Die Abb. 2 zeigt einen quantitativ ausgewerteten, eindimensionalen Orginal-Chromatographiestreifen. Darüber findet sich die zugehörige Extinktionskurve, die sich aus 10 klar getrennten und gut auswertbaren Gauss-Kurven zusammensetzt. Die quantitative Berechnung ergab für 15 Normalratten die in Tabelle 1 zusammengefassten Werte. Die in geringerer Konzentration auf den zweidimensionalen Elektrophero-Chromatogrammen qualitativ nachgewiesenen Stoffe Arginin, Histidin, Methionin, Phenylalanin, Prolin und Tryptophan wurden nicht quantitativ bestimmt.

B. Insulinratten

Das Fleckenmuster der qualitativen Elektrophero-Chromatogramme von Hirnextrakten mit Insulin behandelter Ratten, entsprach insofern dem der Normalratten als

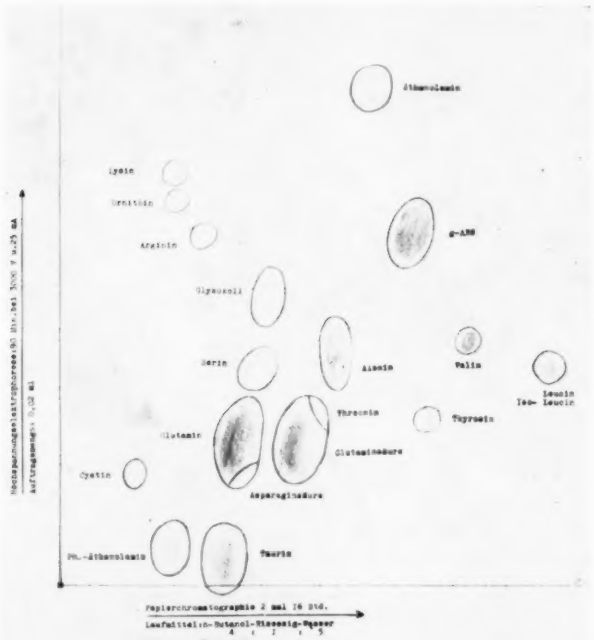

ABB. 1.—Kombiniertes Hochspannungselektropherochromatogramm X = Auftragsstelle. Hochspannungselektrophoretische Trennung bei 3000 v (50 v/cm), 25 mA., 90 Min., Ph 1·9. Danach aufsteigende Papierchromatographie senkrecht zur elektrophoretischen Trennrichtung 2 × 16 Std. in Butanol-Eisessig-Wasser (4:1:5). Färbung mit Ninhydrin nach LEVY und CHUNG (1953). Auftragsmenge 0·02 ml entsprechend dem Extrakt aus 133·3 mg Hirngewebe.

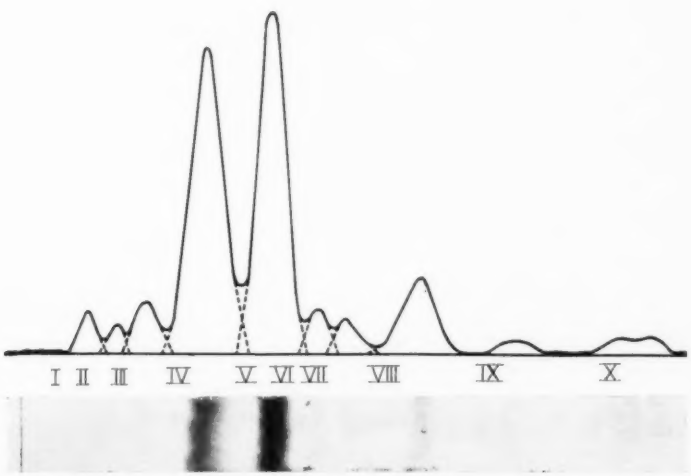

ABB. 2.—Eindimensionaler Chromatographiestreifen der freien Gehirnaminosäuren einer normalen Ratte nach Chromatographie in Butanol-Eisessig-Wasser (4:1:5). Färbung mit Ninhydrin (später zur Haltbarmachung mit Kupfer besprüht). Darüber die zugehörige Extinktionskurve. Auftragsmenge 0·01 ml, entsprechend dem Extrakt aus 66·7 mg Hirngewebe. Man erkennt gut getrennte Gauss-Kurven, die eine exakte quantitative Auswertung erlauben.

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TAB. 1.—MENGEN VERSCHIEDENER FREIER AMINOSÄUREN UND AMINE IN μg/g FRISCHES GEHIRNGEWEBE BEI NORMALEN RATTEN

Spalte 3 Literaturwerte (Angaben folgender Autoren: (1) RICHTER und DAWSON, 1948 (2) KREBS *et al.*, 1949 (3) DAWSON, 1949 (4) DAWSON, 1950 (5) DAWSON, 1951 (6) SCHURR *et al.*, 1950*a* (7) SCHURR *et al.*, 1950*b* (8) AWAPARA *et al.*, 1950*a*, *b* (9) SCHWERIN *et al.*, 1950 (10) CRAVIOTO *et al.*, 1951 (11) TIGERMAN und MACVICAR, 1951 (12) ANSELL und RICHTER, 1954 (13) TALLAN, STEIN und MOORE, 1954 (14) PORCELLATI und THOMPSON, 1957. Die Angaben von TALLAN *et al.* beziehen sich auf das Gehirn der Katze.)

Aminosäure	Mittelwert $\pm \sigma$	Schwankungsbreite	Literaturwerte
Äthanolamin	191·5 ± 7·9	180·0–202·5	207[13]
Alanin	60·8 ± 4·7	52·2–69·8	54[12], 84[13], 135[14]
γ-ABS	360·6 ± 14·7	332·1–384·4	204[12], 234[13], 389[10], 632[8], 810[14]
Asparagins	226·1 ± 19·7	190·8–246·3	297[13], 361[12], 416[10], 451[14], 494[8]
Cystin	12·0 ± 0·7	11·2–12·8	10[13]
Glutamin	553·7 ± 28·2	514·4–600·0	325[10], 500[13], 570[12], 570[9], 598[14], 740[4], 780[5], 790[1], 790[11], 843[2]
Glutaminsäure	1206·9 ± 65·0	1089·5–1321·9	1208[13], 1315[14], 1344[8], 1480[3], 1570[4], 1580[12], 1720[5], 1600[2], 1600[9], 1609[10]
Glykokoll	146·5 ± 11·0	125·0–162·8	95[12], 101[13], 212[14]
Leuc./*Iso*leucin	51·8 ± 2·6	48·5–57·2	18[7], 21[14], 29[6], 30[13]
Lysin/Ornithin	13·5 ± 1·9	10·0–16·5	20[13], 28[6], 31[7] (nur Lysin)
			6[13] (nur Orn.)
P.-Äthanolamin	182·3 ± 16·3	156·0–206·6	171[8], 220[12], 419[13]
Serin	69·7 ± 3·0	64·3–73·9	75[12], 76[13], 135[14]
Taurin	333·9 ± 9·9	318·8–346·5	240[13], 295[8], 360[14], 665[12]
Threonin	42·3 ± 3·7	37·7–48·6	26[13], 79[14], 102[7], 125[6]
Tyrosin	11·0 ± 2·2	8·7–14·6	12[13], 19[14], 21[6], 21[7]
Valin	14·9 ± 0·6	13·8–16·3	12[6], 14[7], 21[13], 30[14]

keine neuen Flecken hinzutraten, oder bekannte Flecken verschwanden. Die Chromatogramme wurden hauptsächlich zur Beurteilung der nicht quantitativ bestimmbaren Spurenaminosäuren wie Arginin, Histidin, Methionin, Phenylalanin, Prolin und Tryptophan angefertigt. Die Fleckengrösse und Farbdichte dieser Substanzen schien im allgemeinen abzunehmen. Jedoch bereitete die Beurteilung mit blossem Auge oft Schwiergkeiten, zumal bekanntlich die Ninhydrinfärbung von Bogen zu Bogen

nicht ganz einheitlich ausfällt. Eine Konzentrationszunahme von Cystin, Serin, Äthanolamin und Phosphoäthanolamin, war schon auf den qualitativen Chromatogrammen meist deutlich erkennbar. Ebenso war stets auch der Cholinfleck grösser und etwas intensiver gefärbt. Die quantitative Bestimmung lieferte dann für die meisten

TAB. 2.—MENGEN VERSCHIEDENER FREIER AMINOSÄUREN UND AMINE IN μg/g FRISCHES GEHIRNGEWEBE BEI 19 IM INSULINKOMA GETÖTETEN RATTEN

Aminosäure	Mittelwert $\pm \sigma$	Schwankungsbreite
Äthanolamin	285·5 ± 25·2	225·0–355·2
Alanin	40·8 ± 7·6	27·0–58·4
γ-ABS	309·5 ± 28·5	250·1–388·7
Asparaginsäure	224·7 ± 28·5	171·0–292·4
Cystin	29·9 ± 4·0	22·5–40·5
Glutamin	361·6 ± 47·4	253·2–468·8
Glutaminsäure	923·9 ± 102·9	720·0–1163·9
Glykokoll	139·8 ± 13·7	112·5–173·1
Leuc./*Iso*leuc.	42·9 ± 7·4	32·6–55·7
Lysin/Ornithin	15·1 ± 4·4	8·4–25·5
P.-Äthanolamin	234·3 ± 21·5	185·9–280·0
Serin	136·0 ± 16·3	112·5–189·0
Taurin	338·8 ± 31·5	248·7–398·1
Threonin	36·3 ± 5·6	25·7–51·2
Tyrosin	11·8 ± 3·2	6·8–16·4
Valin	12·6 ± 2·1	9·4–16·9

Substanzen exakte Werte. Tabelle 2 gibt einen Überblick. Der gefundene Unterschied zwischen Normal- und Schockratten, ist dabei für Äthanolamin, Alanin, γ-Aminobuttersäure, Cystin, Glutamin, Glutaminsäure, Leucin-*Iso*leucin, Phosphoäthanolamin, Serin und Valin statistisch gesichert.* Für Asparaginsäure, Glykokoll, Lysin-Ornithin, Taurin und Tyrosin wurde kein signifikanter Unterschied gefunden.

*Die Signifikanz für den Unterschied der Mittelwerte zwischen Normal- und Kontrollratten wurde nach der Formel

$$t = \frac{x_1 - x_2}{d} \cdot \sqrt{\frac{N_1 \cdot N_2}{N_1 + N_2}} \text{ berechnet}$$

$$\text{Dabei ist } d = \sqrt{\frac{\sigma_1^2(N_1 - 1) + \sigma_2^2(N_2 - 1)}{N_1 + N_2 - 2}}$$

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In Abb. 3 sind die Werte für Normal- und Schockratten in graphischer Darstellung einander gegenübergestellt. Man erkennt sehr deutlich die starke Zunahme von Äthanolamin, Phosphoäthanolamin, Serin und Cystin. Die Abnahme ist bei den in hoher Konzentration vorhandenen Stoffen Glutaminsäure, Glutamin und γ-Aminobuttersäure absolut am grössten. Berücksichtigt man jedoch auch die relative Abnahme in Prozent der Norm und nimmt als Ausgangswert die Mittelwerte der Kontrolltiere, dann ordnen sich die Aminosäuren in folgende Reihe: Asparaginsäure, Glycin, Lysin-Ornithin, Taurin und Tyrosin sind nur wenig oder gar nicht verändert, alle anderen Aminosäuren nehmen ab in steigender Reihenfolge

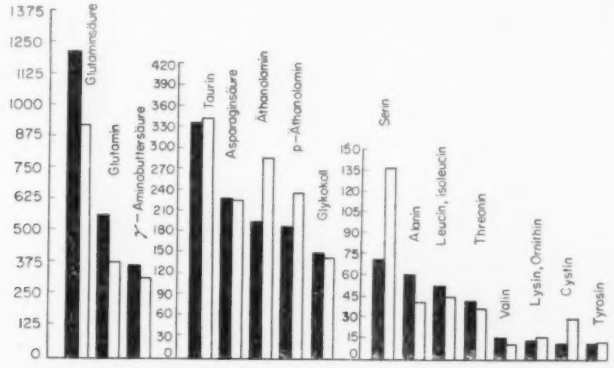

Abb. 3.—Freie Aminosäuren in μg/g Gehirngewebe aus Hirnextrakt von Normal- und Insulinratten in graphischer Darstellung. Schwarze Säulen Normalratten, weisse Säulen Insulinratten. Die Säulen sind nach der absoluten Aminosäuremenge für Normalratten geordnet.

von Glutamin, Alanin, Glutaminsäure, Leucin-*Iso*leucin, Valin, Threonin bis zur γ-Aminobuttersäure. In diese Reihe gehören auch die meisten der nicht quantitativ bestimmten Spurenaminosäuren.

Auch Cholin konnte leider nicht quantitativ bestimmt werden, da die zur Verfügung stehende einschlägige Färbung nur einen ziemlich geringen Kontrast liefert. Ausserdem kam es bei der Chromatographie zu Schwanzbildungen, bei der Elektrophorese zu erheblichen Zonenimperfektionen. Eine exakte Auswertung mit dem Extinktionsschreiber war daher nicht möglich. Wir beobachteten im Insulinkoma stets eine erhebliche Konzentrationszunahme, die sich jedoch nur ungefähr aus der Zunahme von Fleckengrösse und Farbdichte der zweidimensionalen Elektropherochromatogramme abschätzen liess und etwa 50% betragen dürfte.

DISKUSSION

Die Werte sämtlicher von uns bestimmten freien Aminosäuren des normalen Rattengehirns liegen im Bereich der bisherigen Literaturwerte (Tabelle 1). Einige von anderer Seite angegebene Spuren-Aminosäuren (TALLAN *et al.*, 1954; IRREVERRE *et al.*, 1957) haben wir nicht quantitativ bestimmt.

Unterschiede zwischen unseren und fremden Werten können durch die verschiedene Art der Extraktbereitung bedingt sein. Ausserdem sind in den Banden der 16 quantitativ bestimmten Stoffe wahrscheinlich noch andere auf zweidimensionalen Elektropherochromatogrammen qualitativ nachgewiesene Spurenaminosäuren mitenthalten. Bei anderem Vorgehen können sie unter Umständen allein erfasst werden. Daraus müssen sich dann für einzelne Aminosäuren abweichende

Werte ergeben. Ein solcher kleiner Fehler durch nicht abgetrennte Spurenstoffe würde in unseren Chromatogrammen jedoch einheitlich in Erscheinung treten und daher unsere Ergebnisse beim Insulinkoma nicht wesentlich verfälschen.

Der Vergleich des Aminosäurespektrums von Gehirnextrakt mit demjenigen von Liquor und Blutplasma (KNAUFF *et al.*, 1958, 1959, 1960) zeigt eine weitgehendere Übereinstimmung zum Liquor, als zum Plasma. Dies spricht gegen die Existenz einer Hirn-Liquor-schranke für diese Stoffe. Unterschiede zwischen Gehirnextrakt und Liquor, vor allem im Phosphoäthanolamin- und γ-Amino-Buttersäuregehalt, erklären sich wohl dadurch, dass diese Stoffe vorwiegend intracellulär vorkommen.

Bei Betrachtung unserer Werte fällt ihre geringe individuelle Streubreite auf. Im Blutplasma fanden wir mit gleicher Methodik bei Mensch (KNAUFF, DIETERLE und ZICKGRAF, 1959) und Ratte (KNAUFF und WINDSHEIMER, 1960) eine wesentlich grössere Streubreite. Offenbar ist der Gehalt an freien Aminosäuren im Gehirn wesentlich konstanter als im Blutplasma. Auch unter verschiedenen experimentellen Bedingungen scheint er geringeren Schwankungen zu unterliegen als derjenige anderer Organe (THOMPSON *et al.*, 1950; WILLIAMS *et al.*, 1950).

Die Veränderungen nach Insulinüberdosierung sind demgegenüber besonders stark. Ihre Deutung ist jedoch schwierig. *In vitro* werden bei Glukosemangel die meisten Aminosäuren des Hirngewebes verbrannt (WEIL-MALHERBE, 1936, 1952; WALSHE *et al.*, 1958; a.u.b.). DAWSON, der *in vivo* den starken Glutaminsäureabfall im Insulinkoma zuerst beobachtete (DAWSON, 1949; 1950), nimmt einen der Energiegewinnung dienenden Abbau dieser sehr reagiblen Substanz an. Da sie im Gehirn in grösserer Menge vorhanden ist als die Glukose (SCHWERIN *et al.*, 1950), ist ein Zurückgreifen auf diese Reserve durchaus vorstellbar. Das gleiche gilt auch für die beiden anderen, absolut am stärksten abfallenden und mit der Glutaminsäure im Gleichgewicht stehenden (AWAPARA *et al.*, 1950; ROBERTS und FRANKEL, 1950; WEIL-MALHERBE, 1952; ROBERTS *et al.*, 1958) Stoffe Glutamin und γ-Amino-Buttersäure.

Dass die γ-Amino-Buttersäure dabei viel weniger abfällt als die beiden verwandten Stoffe, lässt besondere Mechanismen zur Konstanthaltung dieser physiologisch und pharmakologisch hoch aktiven Substanz (ROBERTS *et al.*, 1958; PURPURA *et al.*, 1958; a. u. b.; TAKAHASHI *et al.*, 1958) vermuten. Ob ihr Abfall während der Insulinhypoglykämie und der damit verbundene Wegfall ihrer Hemmwirkung bei der synaptischen Übertragung mit für das Auftreten der hypoglykämischen Krampftätigkeit verantwortlich ist, lassen wir dahingestellt.

Glycin steht mit Serin, mit Äthanolamin und Cholin im Gleichgewicht, also mit Substanzen, die im Insulinkoma zunehmen. Vielleicht erklärt die Möglichkeit einer laufenden Glycinneubildung aus diesen Stoffen oder aus Glutathion, die im Vergleich zu verwandten Aminosäuren ziemlich geringe Abnahme von Glycin.

Den Abfall der genannten Aminosäuren während der Insulinhypoglykämie durch die Annahme einer vermehrten Durchlässigkeit der Bluthirnschranke und ihre Abwanderung ins Blut zu erklären, halten wir für unwahrscheinlich. Der Übertritt müsste gegen ein Konzentrationsgefälle erfolgen und auch die anderen Aminosäuren in ähnlicher Weise betreffen. Dies ist jedoch nicht der Fall.

Die Konzentration des Taurins, eines vom Gehirn nicht weiter abbaubaren Spaltproduktes des Cystinstoffwechsels bleibt gleich. Es kommt als energielieferndes Substrat nicht in Frage. Die fehlende Asparaginsäureverminderung ist schwieriger zu erklären. CRAVIOTO und Mitarbeiter (1951), fanden im Insulinkoma sogar einen Anstieg und machten eine Aktivierung der Transaminierungsreaktionen dafür verantwortlich. In den in vitro Versuchen von WALSHE *et al.* (1958*b*) fiel die Asparaginsäure ebenfalls am wenigsten ab. Er nimmt an, dass über die Asparaginsäure andere

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Aminosäuren in den Zitronensäurecyclus eingeschleusst werden, und sie dabei selbst am längsten übrig bleibt. Dies ist möglich, jedoch ist dann der Grund für den von uns beobachteten Unterschied zum Verhalten der ähnlich gebauten und mit gleicher Funktion ausgestatteten Glutaminsäure nicht ganz klar. Tyrosin ist wahrscheinlich durch seinen schwerer spaltbaren Benzolring vor schnellem Abbau geschützt.

Bei Serin, Äthanolamin, Phosphoäthanolamin und Cholin, deren Konzentration während der Insulinhypoglykämie ansteigt, handelt es sich um Bausteine von Phosphatiden, also von Stoffen, die im Insulinkoma (PAGE *et al.*, 1931; RANDALL, 1940; MCGHEE *et al.*, 1951), oder sonst bei vermindertem Glukoseangebot, abgebaut und wahrscheinlich vom Gehirn als energielieferndes Substrat verwendet werden (ABOOD und GEIGER, 1955). Dabei kommt es offenbar zum Freiwerden und zur Anhäufung niedermolekularer Bruchstücke*.

Allerdings lässt sich über das absolute Ausmass ihrer Freisetzung allein aus der Bestimmung der freien Gehirnaminosäuren nichts aussagen, da nicht bekannt ist, eine wie grosse Menge der entstehenden Spaltstücke sofort weiter verbrannt oder umgebaut wird. Auch ist ausser mit einem Lipoidabbau wahrscheinlich mit einem gewissen Abbau von Proteinen zu rechnen (ABOOD und GEIGER, 1955). Dabei könnten zusätzliche Aminosäuren frei werden†.

Cystin ist die einzige nicht am Aufbau der Phosphatide beteiligte Aminosäure, die im Insulinkoma stark ansteigt. Möglicherweise stammt sie aus dem im Gehirn reichlich vorhandenen (TALLAN, STEIN und MOORE, 1954), aber von uns nicht bestimmten Glutathion. Seine Menge würde dafür ausreichen. Die bei einem angenommenen Glutathionabbau ebenfalls freiwerdenden Aminosäuren, Glutaminsäure und Glycin, dürften sofort weiter abgebaut werden, oder entziehen sich im viel grösseren 'pool' dieser Aminosäuren dem Nachweis.

Mengenmässig wird die Zunahme der genannten Substanzen durch die gleichzeitige Abnahme der anderen Stoffe kompensiert oder übertroffen, wenn man dabei von der Glutaminsäure absieht. Wird daher nur diese einzeln, die übrigen Aminosäuren aber gemeinsam als freier α-Amino-Stickstoff kolorimetrisch bestimmt, dann muss der Eindruck enstehen, allein die Glutaminsäure sei abgefallen, alle übrigen Aminosäuren aber gleich geblieben. Wahrscheinlich ist so das Ergebnis von DAWSON (1950) zu

**Anm. bei der Korrektur:*

Nach Einreichen unserer Arbeit wurden wir auf eine Veröffentlichung von G. B. ANSELL und S. SPANNER (1959 *J. Neurochem.* **4**, 325) aufmerksam gemacht, nach der während der Insulinhypoglykämie der Gehalt an freiem Phosphoäthanolamin etwas absinkt, weil die ATP-abhängige Synthese aus Äthanolamin und Phosphorsäure herabgesetzt ist. Die Autoren verwendeten jedoch meist kleinere Insulindosen und liessen die Ratten wesentlich kürzere Zeit überleben. Vielleicht ist dann ausschliesslich die Neusynthese von Phosphoäthanolamin gestört, während noch nicht mit einem Abbau von Gehirnphosphatiden zu rechnen ist, der den Spiegel des freien Phosphoäthanolamins sekundär wieder erhöhen würde.

Nach hohen Insulindosen und gleichzeitiger Gabe von radioaktivem ^{32}P-Phosphat müsste bei der Beurteilung der spezifischen Aktivität der Phosphoäthanolaminfraktion mit einem Verdünnungseffekt durch nicht radioaktives, aus dem Kephalinabbau stammendes Phosphoäthanolamin gerechnet werden. Das gleiche gilt auch für die anderen P-haltigen Fraktionen.

†*Anm. bei der Korrektur:*

Inzwischen haben wir in weiteren Versuchen auch die Kephaline und die Proteine des Rattengehirns getrennt gewonnen und nach Säurehydrolyse ihre Ninhydrin-färbbaren Bausteine quantitativ bestimmt. Nach fünfstündiger Insulinhypoglykämie ergab sich ein Abfall an Phosphatidyl-Serin und Phosphatidyl-Aethanolamin von etwa 10 Prozent. Der absolute Zahlenwert pro Gramm Hirngewebe entsprach fast genau dem beobachteten Zuwachs an freiem Serin und freiem Aethanolamin und Phosphoaethanolamin. Die Aminosäuren der Proteinfraktion waren dagegen im Insulinkoma nicht signifikant verändert. Ein nennenswerter Abbau von Gehirnproteinen während der Insulinhypoglykämie ist daher sicher auszuschliessen.

Wir werden über diese Versuche in Kürze gesondert berichten.

erklären, der über einen Abfall lediglich der Glutaminsäure bei Gleichbleiben des Gesamt-Amino-Stickstoffs berichtet.

Insgesamt scheinen unsere Ergebnisse die Ansicht zu stützen, dass Nervengewebe im Notfall auch andere Stoffe als Glukose, das physiologische Substrat seines oxydativen Stoffwechsels (HIMWICH und NAHUM, 1932; KETY *et al.*, 1947; MCILWAIN, 1958) zur Energielieferung heranziehen kann, Eine Vielzahl experimenteller Befunde (HOLMES *et al.*, 1930; SCHMIDT *et al.*, 1945; HYDEN, 1947; KETY *et al.*, 1947; GEIGER und MAGNES, 1947; GEIGER *et al.*, 1952; MULLINS, 1953; STONE, 1955; GERARD, 1955; ALLWEIS und MAGNES, 1956; ALLWEIS und MAGNES, 1958; ERBSLÖH, BERNSMEIER und HILLESHEIM, 1958; u.a.) spricht heute für diese Ansicht. Da die Bluthirnschranke eine schnelle Aufnahme anderer energieliefernder Substrate aus dem Blut weitgehend ausschliesst, scheint im Insulinkoma der Rückgriff auf bereits im Gehirn vorhandene, sonst als Strukturbestandteile angesehene Stoffe erforderlich zu sein.

Ob alle von uns beobachteten Veränderungen ein spezifischer Effekt der Insulin-Hypoglykämie sind, oder ob sie zum Teil sekundär durch die mit erheblich grösserem Energiebedarf einhergehende Krampftätigkeit verursacht werden, vermögen wir nicht zu entscheiden. Dazu wären Versuche mit Krampfgiften, mit Elektroschock, mit verschiedenen Fermentblockern usw. erforderlich. Dass der Abbau von spezifischen Strukturbestandteilen des Gehirns, gleich auf welche Weise er nun entsteht, für dieses nicht belanglos sein kann, sondern von einem gewissen Grad an zu irreversiblen oder schwer reparablen Schäden führt, ist einleuchtend.

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ZUSAMMENFASSUNG

(1) Im Hirnextrakt von normalen und von im Insulinkoma getöteten Ratten wurde freies Äthanolamin, Phosphoäthanolamin und 14 verschiedene Aminosäuren nebeneinander quantitativ bestimmt. Sechs weitere Aminosäuren, Cholin und einige unbekannte Substanzen wurden qualitativ nachgewiesen. Unsere Werte stimmen gut mit den Literaturwerten überein.

(2) Bei normalen Ratten fand sich für sämtliche untersuchten Stoffe eine äusserst geringe individuelle Streubreite. Das Aminosäurespektrum des normalen Gehirns ist danach ziemlich konstant. Es gleicht weitgehend dem des Liquors, unterscheidet sich jedoch von dem des Blutplasmas. Dies spricht gegen die Existenz einer eigenen Hirn-Liquorschranke.

(3) Im Insulinkoma wurde ein erheblicher Anstieg der Phosphatidbausteine Serin, Äthanolamin, Phosphoäthanolamin und Cholin beobachtet. Dies spricht für einen Phosphatidabbau während der Insulinhypoglykämie. Ferner war freies Cystin vermehrt.

(4) Die meisten übrigen Aminosäuren fallen während der Insulinhypoglykämie stark ab. Wahrscheinlich werden sie an Stelle der Glukose metabolisiert. Ihr Abfall kompensiert zum Teil den Anstieg der Phosphatidbausteine Serin, Äthanolamin und Phosphoäthanolamin, sodass sich der Gesamt-Amino-N wenig ändert.

Anerkennung—Wir danken der Deutschen Forschungsgemeinschaft für die Gewährung einer Sachbeihilfe, die die Durchführung der Arbeiten ermöglichte.

SUMMARY

(1) Ethanolamine, phosphoethanolamine and 14 amino acids have been determined in brain extracts from normal rats and from rats killed in insulin coma. Six

other amino acids, choline and some unknown substances were demonstrated qualitatively. Our results agree with those in the literature.

(2) In normal rats, there was very little individual variation for those substances investigated. The amino acid pattern of the normal brain is fairly constant, resembling to a large extent that of the cerebrospinal fluid, but differing from that of the blood plasma. This is evidence against the existence of a separate fluid pool in the brain.

(3) A considerable rise in phosphatide components, namely serine, ethanolamine, phosphoethanolamine and choline, was observed in insulin coma. This indicates a degradation of phosphatides during insulin hypoglycaemia. There was also a rise in free cystine.

(4) Most of the remaining amino acids decrease markedly during insulin hypoglycaemia, being probably metabolized in place of glucose. Their decrease compensates in part for the rise in the phosphatide components, serine, ethanolamine and phosphoethanolamine; there is consequently only a slight alteration in the total amino-N.

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SOME METABOLIC CHANGES ACCOMPANYING LEÃO'S SPREADING CORTICAL DEPRESSION IN THE RAT

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IN 1944 LEÃO described a particular electrical reaction of the cerebral cortex to stimuli acting directly on its surface. Electrical, mechanical and chemical stimuli cause a local decrease in amplitude of the spontaneous EEG. This decrease spreads slowly at a rate 3–6 mm/min in all directions over the whole cortex. Complete recovery of the original activity occurs after from 10 to 20 min. The front of the wave of spreading depression is accompanied by a striking slow potential change. The initial surface negativity, attaining 5–10 mv in amplitude, is replaced after 1–2 min by a lower wave of longer lasting positivity. A new wave can be produced from the same focus after a minimum recovery period of 4–5 min (LEÃO, 1944).

Since the discovery of this phenomenon various aspects of it have been studied (see recent review by MARSHALL, 1959). The electrophysiological properties of spreading depression are already well known but the mechanism of initiation and spreading is still obscure. Some properties of spreading depression seem to indicate that metabolical changes are involved: (1) The rate of spreading is slow; (2) the slow potential change is temperature dependent with a $Q_{10} = 1{\cdot}8$ (BUREŠ, 1956); (3) the decrease of cortical pO_2 during spreading depression is not caused by vasoconstriction, as was assumed by VAN HARREVELD and STAMM (1952), but rather by increased oxygen utilization due to vasodilatation (BUREŠOVÁ, 1957); (4) the analogy between the initiation of spreading depression *in vivo* and the increase in respiration and aerobic glycolysis of cerebral cortex slices *in vitro* (BUREŠ, 1956). A decrease in glycogen in the rat brain during spreading depression has been observed (KŘIVÁNEK, 1958).

In the experiments reported in the present paper a more precise correlation between electrical and chemical changes has been attempted.

METHODS

Experiments were performed on rats weighing 150–200 g and anaesthetised with Dial (40 mg/kg i.p.). Trephine openings (5 mm in diameter) were made above both hemispheres. Spreading depression was elicited by the application of 25% KCl solution into a small trephine opening (C in Fig. 1.) in front of a large one. The slow potential change of spreading depression was recorded with wick cell calomel electrodes A and B (Fig. 1) against a reference electrode above the control hemisphere. The steady potential shifts were amplified with a chopper amplifier and recorded with an ink-writing EEG apparatus. Spatial distribution of the negative wave was determined from the recordings of A and B. In the middle part of Fig. 1, the distribution of the steady potential on the cortical surface in a parasagital plane is reconstructed from the records of A and B at the moment of the freezing of the brain tissue. The onset of negativity at electrode A indicates that the negative wave is passing through the part of cortex between electrodes A and B (Fig. 1–I). Maximal negativity under electrode A indicates that the area between the electrodes displays various degrees of repolarization while in the more occipital regions depolarization is still increasing (Fig. 1–II). Zero potential difference between

electrode A and the reference electrode indicates that the steady potential of the experimental hemisphere has returned to normal (Fig. 1–III).

Brain samples were obtained in each of these three situations. The rats were killed by immersion in liquid nitrogen. The skull was removed from the frozen head and the hemispheres were separated by a sagital cut using a steel saw rotating in liquid nitrogen. In each hemisphere the focus of the spreading depression was eliminated by cutting out that part of the tissue to which KCl solution had been applied. The hemispheres were cut into two parts at a line corresponding approximately to the level of electrode A. The head was fixed in a holder and moved towards the saw which was rotating in a plane parallel to the cortical surface. A thin slice (1·5 mm) of cortex, almost exclusively, was obtained. The width of the slice could be precisely adjusted.

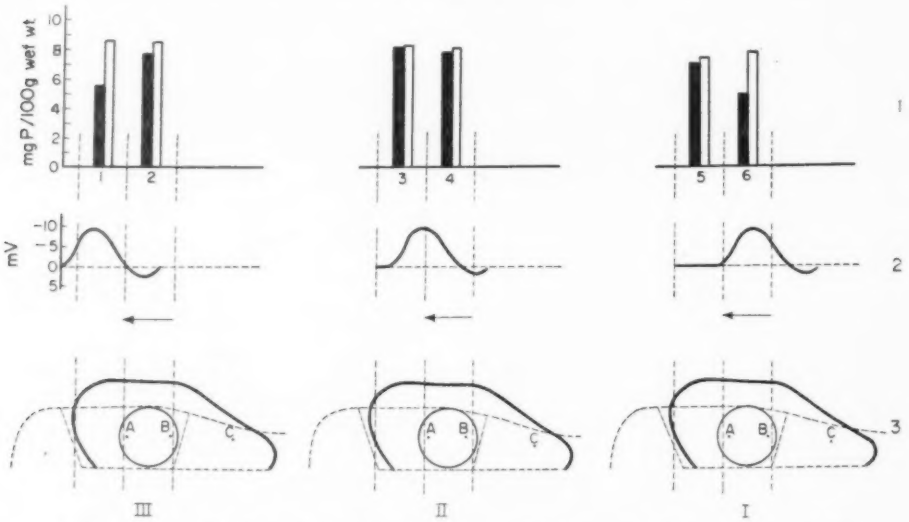

FIG. 1.—Changes in creatine phosphate level in the cerebral cortex of rats corresponding to the various phases of the negative wave of spreading depression shown in 2. Black columns represent experimental values and white columns the corresponding control values. Differences obtained in 1 and 6 are statistically significant for $P < 0{\cdot}01$. 3. Diagrammatic representation of the positions of the electrodes (A, B) in relation to the point of application of KCl (c).

The pieces of brain tissue were stored under liquid nitrogen. After weighing on a torsion balance in a cold room at 0–1°, the frozen slice was powdered in a mortar and extracted with ice-cold 10% TCA for estimation of lactic acid and phosphates, or treated with 0·3 N-$Ba(OH)_2$, water and 5% $ZnSO_4 . 7H_2O$ for the determination of glucose.

Lactic acid was determined according to HULIN's and NOBLE's (1953) modification of the method of BARKER and SUMMERSON (1941). Inorganic phosphate, creatine phosphate and ATP were determined according to ERNSTER *et al.* (1950). Glucose was determined either as total reducing substances (SOMOGYI, 1952) after deproteinization or directly as glucose after separation by paper chromatography using the method described by GEY (1956) with slight modifications.

In some instances the whole hemisphere was analysed. Spreading depression was elicited from a small trepine opening above the occipital cortex. The animals were killed at varying intervals after the application of KCl solution. The hemispheres were separated in a frozen state and were prepared for chemical analysis as described above. (For details see KŘIVÁNEK, 1958).

Spreading depression evoked in one hemisphere by the local application of KCl does not pass to the opposite one, which can be used as a control (BUREŠ and KŘIVANEK 1959) Use was made of this for the statistical treatment of the results. Differences between paired values from the corresponding brain regions from individuals were calculated. Student's '*t*'-test was used for evaluation of the statistical significance and the results are expressed as mean values ± the standard error of the means (SEM).

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RESULTS

Changes in glucose level during spreading depression. Fig. 2 shows changes in glucose level corresponding to various phases of the slow negative potential wave. In the cortical area of maximal negativity, the glucose level had decreased by 37 per cent; 1, 3 and 5 min after complete repolarization (see Methods) the glucose level

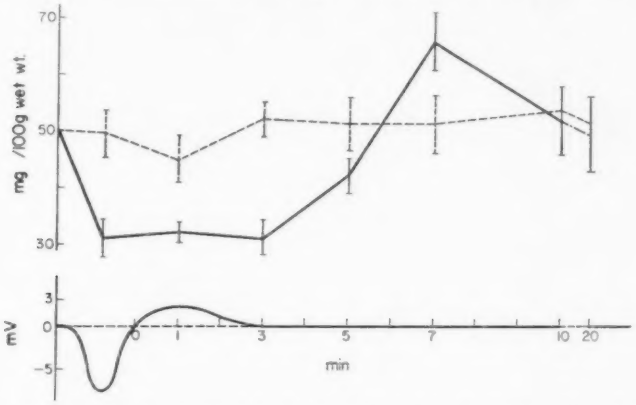

FIG. 2.—Changes in glucose level in the cerebral cortex of rats at various intervals after passage of a single wave of spreading depression. (shown in the lower part of the Figure).
——— experimental values
- - - - - - control values

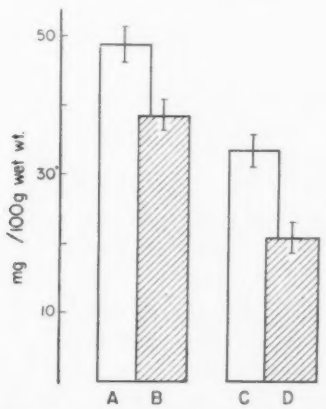

FIG. 3.—Difference between glucose determined as total reducing substances (A, B) and after chromatographic separation (C, D). White columns: control values, shaded columns: values after 30 min lasting spreading depression evoked by topical application of 25% KCl.

was still below normal, by 24, 40 and 17 per cent respectively. A rapid increase of glucose then occurred and the value at the 7th min was 28 per cent higher than in the control hemisphere possibly due to overcompensation. After 10 min the original glucose level was again reached.

In order to establish whether or not the changes in total reducing substances gave a true value for the changes in the glucose content, they were compared with the values obtained by the direct determination of glucose after chromatographic separation. It can be seen from Fig. 3 that the glucose level in the control hemisphere was 30 per

cent lower when determined chromatographically than when determined as total reducing substances. This is in general agreement with GEY's (1956) findings that glucose represents only 40 per cent of the total reducing substances in brain. Further, it is clear from Fig. 3, that the changes in reducing substances accompanying spreading depression are due to changes in the glucose content, as a similar decrease was obtained when glucose was determined after chromatographical separation.

The changes in the glucose level of the whole hemisphere and of the cortical slices are summarized in Table 1. In the cortical region in which the wave of negativity

TABLE 1.—CHANGES IN GLUCOSE AND LACTIC ACID LEVELS DURING SPREADING DEPRESSION (S. D.)

	Whole hemisphere						Cortical slabs				
Substrate analysed	No. of rats	Duration of S.D. (min)	Control hemisphere (mg% ± SEM)	Exp. hemisphere (mg% ± SEM)	Difference between paired values (%)	Difference between paired values (mg% ± SEM)	No. of rats	Control hemisphere (mg% ± SEM)	Exp. hemisphere (mg% ± SEM)	Difference between paired values (%)	Difference between paired values (mg% ± SEM)
Lactic acid	6	10	21·4 ± 4·4	28·4 ± 4·8	+37	8·0 ± 1·2 $P < 0.01$	14	22·0 ± 4·2	44·4 ± 4·4	+102	22·4 ± 3·9 $P < 0.01$
	6	30	20·4 ± 4·6	28·6 ± 4·4	+41	8·3 ± 2·2 $P < 0.05$					
Glucose	10	30	49·6 ± 2·4	38·8 ± 2·2	−22	10·8 ± 2·8 $P < 0.01$	14	49·5 ± 4·2	31·0 ± 3·2	−37	18·5 ± 2·6 $P < 0.01$

attains its maximum, glucose is reduced to approximately 63 per cent of its value in the corresponding control cortex. When glucose was determined in the whole hemisphere after passage of more than one wave i.e. 30 min after application of KCl, the decrease was only 22 per cent. This was presumably due to the fact that spreading depression is confined to the cortex. Other parts of the hemisphere remain unaffected and do not, therefore, contribute to the metabolic response. In addition the glucose level can partially recover during the intervals between individual waves of negativity (Fig. 2).

Changes in lactic acid during spreading depression (*Table* 1). In the cortical region in which the wave of negativity attains its maximum, the lactic acid content is approximately twice that in the corresponding control. The increase in the lactic acid content of the whole hemisphere 10 and 30 min after KCl application was 37 and 41 per cent respectively.

Changes in creatine phosphate level during spreading depression. It is known from *in vitro* experiments (MCILWAIN, 1952; HEALD, 1954; SUGAWARA and WADA, 1957) that an increased concentration of potassium ions in the incubation medium of cerebral cotrex slices causes a rapid fall in creatine phosphate level. In experiments with reversible ischaemic depolarization of cerebral cortex (KŘIVÁNEK, BUREŠ and BUREŠOVÁ, 1959) a close correlation between recovery of cortical polarity and recovery of creatine phosphate level was shown. In the present experiments the creatine phosphate level was determined for various phases of the slow potential wave. The results are shown schematically in Fig. 1. In the region of maximal negativity the level of creatine phosphate was 37 per cent lower then in the control hemisphere. During repolarization the creatine phosphate level rapidly attains the normal value. No change has been found at the beginning of depolarization. It seems, therefore, that a detectable decrease of creatine phosphate occurs only in a very narrow region of cerebral cortex corresponding to the maximum of the negative wave. This result is in agreement with the observation that the recovery of the creatine phosphate level after short-lasting

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anoxia (10 and 20 min) precedes to some extent the recovery of cortical polarity (KŘIVÁNEK, BUREŠ and BUREŠOVÁ, 1959).

The failure to detect a clear-cut change when the negativity maximum was between the two compared regions might be explained in the following way: a depolarization of 1 mv corresponds to a breakdown of approximately 0·25 mg per cent of creatine phosphate. Only a small part of the analysed tissue slice is in some degree of depolarization. The average change of creatine phosphate is therefore very small. As a result of the transverse cutting, a band of tissue about 1 mm wide is lost. This may well correspond to the zone of maximal negativity.

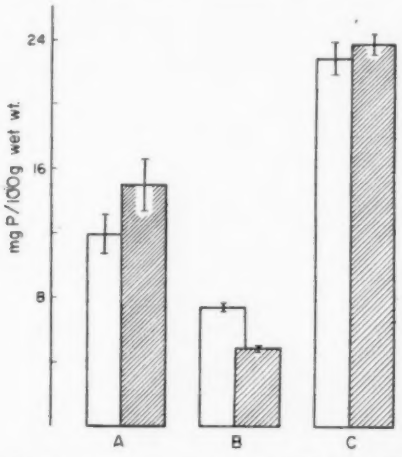

FIG. 4.—Inorganic phosphate (A), creatine phosphate (B) and ATP (C) in the cerebral cortex in the moment of the passage of the negativity maximum of the slow potential wave (shaded columns) and in control cortex (white columns).

Changes in inorganic phosphate and ATP in the cortical region of maximum negativity are compared with those of creatine phosphate in Fig. 4. No changes in ATP level were found corresponding, for example, to the findings of SUGAWARA and WADA (1957). Inorganic phosphate appears to increase proportionally to the fall in creatine phosphate. The great variation of individual values may have been due to contamination of the slices with powdered bone while dissecting the tissue from the frozen skull.

DISCUSSION

Some chemical manifestations of spreading depression have been studied by the usual analytical and preparative methods. It was possible to detect a decrease in the glycogen level of the whole hemisphere after evoking spreading depression by topical application of 2 or 25 per cent solutions of KCl (KŘIVÁNEK, 1958). The return of glycogen to normal after passage of a single wave was slow and the accumulation of the effects of individual waves resulted in a relatively large change in the glycogen level. For substances which recover more rapidly after passage of a single slow potential wave it is more suitable to analyse the brain tissue directly affected by the spreading depression and to eliminate those parts of the hemisphere which are unaffected (noncortical parts). For instance, the decrease in glucose and the increase in lactic acid accompanying the spreading depression were greater in cortical slices than

in the whole hemisphere. Changes in creatine phosphate during spreading depression were undetectable in the whole hemisphere (ZACHAR and RUŠČÁK, 1958).

Various mechanical, chemical and electrical stimuli are able to evoke spreading depression and give the same metabolic responses (KŘIVÁNEK, 1958). All these agents may act in the same manner, possibly by releasing some active substance which spreads the reaction to neighbouring neurons.

From the list of substances evoking spreading depression only potassium ions and some amino acids (glutamate and aspartate) occur naturally in cerebral tissue. Several experimental findings support the idea that potassium is the transmitting substance: (1) GRAFSTEIN (1956) was able to influence the velocity of spreading depression by applying direct current across an isolated slice of cat cortex. The wave of negativity advanced at a faster rate towards the negative pole than towards the positive pole, suggesting that a positively charged particle might be the transmitter. (2) By washing the cortical surface through an epidural fistula a significant increase of potassium flux was found, both in the focus of the spreading depression and in area through which the wave of negativity passed (BUREŠ and KŘIVÁNEK, 1959). (3) The fall in the conductivity of cerebral tissue (VAN HARREVELD and OCHS, 1956), the swelling of cortical neurons (VAN HARREVELD, 1958) and the accumulation of chloride in brain apical dendrites (VAN HARREVELD and SCHADÉ, 1959) support the idea that an important electrolyte shift occurs during spreading depression. VAN HARREVELD (1959) has suggested that either glutamic acid and/or aspartic acid is responsible for the spreading of the depressions, since they evoke spreading depression at lower concentrations than does potassium. If this were so, the explanation given above of the experiments of GRAFSTEIN would not be valid as glutamic and aspartic acids would move towards the positive pole. On the present evidence it is difficult to decide between the suggestions. It is possible that both potassium and glutamate are involved. The interrelationship of potassium ions and glutamate in the transport of water and electrolytes across the cell membrane in brain and retina is well known (KREBS *et al.*, 1949; PAPPIUS and ELLIOTT, 1956; AMES, 1956).

The chemical changes accompanying spreading depression are very similar to those evoked by anoxia or ischaemia. The decrease of pO_2 occurring in brain cortex during spreading depression (VAN HARREVELD and STAMM, 1952) is accompanied by vasodilatation (BUREŠOVÁ, 1957) and is, therefore, due not to a decrease in the oxygen supply but to an increased O_2 consumption. Changes in glucose, lactic acid and creatine phosphate levels, and the increased respiration are similar to those found in cerebral cortex slices after increasing the potassium ion concentration of the medium (ASHFORD and DIXON, 1935; MCILWAIN, 1952; SUGAWARA and WADA, 1957).

It is difficult to say at present whether the metabolic changes found in these experiments are the cause or the consequence of spreading depression. It is not yet clear whether agents evoking spreading depression affect primarily the normal metabolism of cerebral tissue and in this way reduce the ability of cortical neurones to maintain normal membrane polarity, or whether the metabolic changes are due to increased requirements on the energy-providing mechanisms after initial depolarization.

SUMMARY

The changes of glucose, lactic acid and creatine phosphate levels during the spreading depression of LEÃO were studied either by analysis of the whole hemisphere after

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passage of many waves of spreading depression or by analysis of the slices of cerebral cortex taken during different phases of the wave. In the former instance the increase in the lactic acid level (40 per cent) and the decrease in the glucose level (20 per cent) is smaller than in the latter (101 and 37 per cent respectively). Changes in creatine phosphate were closely connected to the degree of depolarization accompanying spreading depression. In the area of maximal negativity creatine phosphate was decreased by 37 per cent. Recovery of creatine phosphate level preceded complete repolarization.

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HISTOCHEMICAL INVESTIGATIONS ON SUCCINIC DEHYDROGENASE IN THE CENTRAL NERVOUS SYSTEM—V

THE DIENCEPHALON AND BASAL TELENCEPHALIC CENTRES OF THE GUINEA PIG

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THE present article completes the detailed description of the nuclear pattern of the guinea pig's brain as defined by a histochemical demonstration of succinic dehydrogenase activity (SDA); the diencephalon and the subcortical telencephalic nuclei are described. Particular attention is focussed on the enzyme content of the thalamic nuclei as related to their cortical projection areas; the gradations of SDA among areas of cerebral cortex have been described in a preceding article (FRIEDE, 1960).

The nomenclature of the thalamus refers to the description of the guinea pig thalamus by HESS (1955); this article should be consulted for cross reference of the nomenclature of thalamic nuclei. The anatomy of the amygdaloid nucleus and certain basal telencephalic centres of the guinea pig were described by JOHNSON (1957).

MATERIAL AND METHODS

Nine complete series of the diencephalon of young adult guinea pigs were used for this investigation. Eight of the series were cut frontally and the ninth was cut fronto-occipitally and parallel to the basis of the skull. The histochemical technique was identical with that used in the preceding investigations, but Nitro BT (NACHLASS *et al.*, 1957) was employed to a larger extent. Frozen sections, 60 μ thick, were cut from unfixed tissue and were incubated for 2 hr at 38° in a medium containing 0·25% Tetrazolpurpur, 1% sodium succinate, and 0·1 M-phosphate buffer, pH 7·8. An incubation time of 90 min was used for Nitro BT. The sections were then fixed in formalin and mounted with glycerin gelatin.

Densitometric measurements of the gradations of the histochemical reaction were made in one additional series stained with Nitro BT in which thickness of sections, temperature, timing of the incubation period, conditions of mounting, etc., were kept constant with the greatest possible care. The measurements were taken on the screen of a Zeiss Ultraphot II by means of a Welch Densichron densitometer equipped with a blue CE-34 phototube. Thirty-seven representative regions were measured; the data were subjected to statistical analysis and were expressed in extinction values (E) as a percentage of the putamen value. The putamen shows considerable gradations of SDA in its neuropil (FRIEDE, 1960); therefore, a region in the head of the caudate nucleus was measured which represented most closely the average activity. Generally, measurements were taken from the most homogeneous part of a nucleus; in a few nuclei with unhomogeneous structure, the measurements were taken from the darkest regions. As a means of control, measurements were also taken from the upper layers of the postcentral cortex: their E 111 $\pm$ 8 compares well with the E 104 reported for this region in the preceding article. The size of the standard deviation is a fairly reliable parameter

of the homogeneity of the structure of the neuropil. Nuclei with a reticular neuropil, for example, always show large standard deviations; a deviation of ±19, therefore, does not imply unreliability of the technique.

The verbal description of the gradations of SDA was standardized according to the measurements: weak SDA was defined as below 30, mild SDA as 30–60, strong SDA as 60–90, and very strong SDA as above 90.

RESULTS

Putamen and Nucleus Caudatus (CAU)

Both nuclei are characterized by very strong SDA (E 100 ± 13) distributed homogeneously throughout their neuropil; the perforating fibre bundles are sharply delineated (Fig. 6, 9); but the large nerve cells are barely discernible from the neuropil. Photometric measurements show considerable gradations of SDA among parts of the nucl. caudatus; the transitions, however, are so gradual that a subdivision is not possible except in the part adjacent to the amygdaloid nucleus which shows a considerable decrease of SDA (E 61 ± 7). A narrow layer of weak SDA separates the neuropil of the nucl. caudatus from the ependyma.

Pallidum (PA)

The pallidum shows strong SDA in its nerve cells but mild SDA (E 39 ± 5) in a scarce and reticular neuropil; the boundary between putamen and pallidum is demarcated by a sharp decrease of SDA in the neuropil of the latter (Fig. 6).

Claustrum (CL)

The claustrum shows weak SDA (E 29 ± 5); its neuropil blends with that of the adjacent deep cortical layers which also show weak or mild SDA. A mild SDA prevails in the neuropil in the dorsal part of the claustrum while the ventral part shows nerve cells clearly discernible from a neuropil of weak SDA (Fig. 3).

Thalamus

Most of the thalamic nuclei show characteristic enzyme patterns and some are more conspicuous histochemically than by cell stains. Histochemical similarities among nuclei establish a gross thalamic organization which differs from HESS's cytoarchitectonic grouping of nuclei. In the 'inner nuclear group' weak or mild SDA prevails in the neuropil. These nuclei are bordered laterally and ventrally by the U-shaped 'inner lamina' of nuclei which shows SDA predominantly in cell bodies. An 'outer nuclear group' shows SDA predominantly in the neuropil and some in cell bodies which are, however, barely discernible from the neuropil. The 'outer nuclear group' is encircled by the 'outer lamina', which shows SDA in cells and very little in the neuropil (Fig. 1E).

At medial levels of the thalamus, the 'inner nuclear group' is formed by the nucl. paraventricularis, paratenialis, parts of the nucl. paracentralis, and the medial nucleus. The 'inner lamina' is formed by (from dorsolateral to medioventral) a small cell group between the medial and lateral nucleus, not discerned by HESS, the ventral dorsal medial nucleus, and parts of the paracentral, central, and rhomboid nuclei. The 'outer nuclear group' includes the lateral nucleus, ventral nucleus, and ventral medial nucleus. The 'outer lamina' is formed by the nucl. reticularis thalami. This subdivision is less accentuated in the anterior and posterior part of the thalamus. The enzyme content

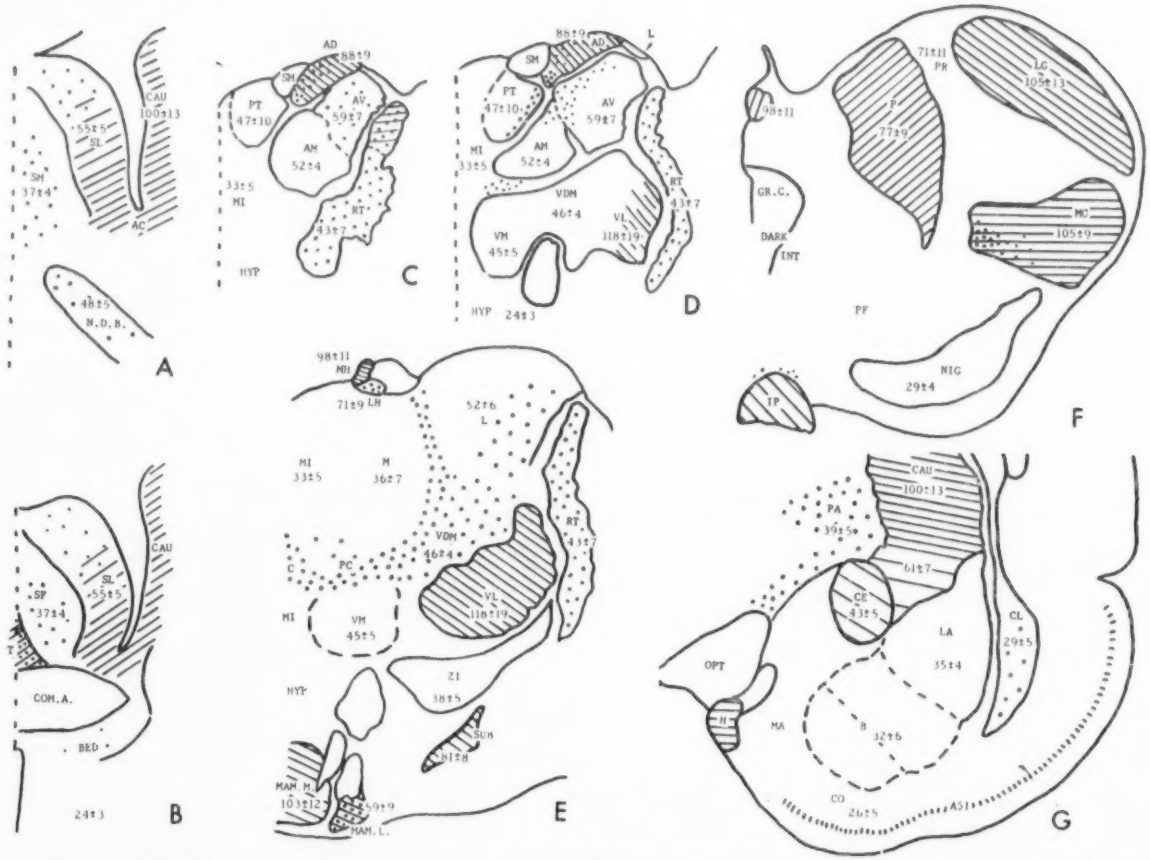

FIG. 1.—Schematic drawings of the regions investigated including the measurements of succinic dehydrogenase activity. The dots and rings indicate prevalence of enzymic activity in the perikarya over that in the neuropil. A collection of all the abbreviations used here and in the plates is found at the end of the paper.

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of the perikarya composing both laminae appears to change *pari passu* with that of the neuropil of adjacent nuclei.

Anterior nuclear group (Fig. 2)

Anterior dorsal nucleus (*AD*). Strong SDA (88 ± 9) distributed diffusely throughout the neuropil characterizes this nucleus. Large, intensely stained nerve cells are discernible from the neuropil. The medio-ventral pole of the nucleus shows a well defined, small cell group with strong SDA in the perikarya but none in the neuropil (Fig. 1D).

Anterior ventral nucleus (*AV*). Its neuropil is delineated from the anterior dorsal nucleus by a decrease of SDA (E 59 ± 7); nerve cells with strong SDA are readily discernible from the neuropil.

Anterior medial nucleus (*AM*). This nucleus shows mild SDA (E 52 ± 4) distributed diffusely throughout its neuropil; no nerve cells are discernible. The distribution of SDA resembles that in the adjacent parataenial nucleus.

Lateral nuclear group

Reticular nucleus (*RT*). The reticular nucleus resembles the pallidum showing strong SDA in nerve cells and their short dendrites and mild SDA (E 43 ± 7) in a scarce, reticular neuropil (Fig. 4). There are slight regional gradations of the compactness of the neuropil; the latter is extremely dense at the rostral-dorsal pole appearing like an individual 'subnucleus' (Figs. 1C and 2).

Lateral nucleus (*L*). The lateral nucleus is characterized by mild SDA (E 52 ± 6) in its neuropil; large nerve cells are readily discernible by stronger SDA. The ventral dorsal medial nucleus is similar to the lateral nucleus but the contrast between perikarya and neuropil is less accentuated in the latter.

Ventral nuclear group

Ventral lateral nucleus (*VL*). This nucleus is conspicuous, even macroscopically, for its very strong SDA (E 118 ± 19). The neuropil is perforated by abundant small fibre bundles and shows a tendency to form irregular clusters of very strong SDA, particularly in the central part of the nucleus where the measurements were taken (Fig. 4). Large, intensely stained nerve cells can be discerned in regions with less SDA in the neuropil. The nucleus has sharp borders dorsomedially but a few small cell groups with a similar distribution of SDA are found farther dorsally. It is not clear whether these groups represent dislocated parts of the nucleus or separate 'nuclei'. The rostral part of the ventral lateral nucleus shows weaker SDA and its neuropil is more homogeneous than that of the caudal part of the nucleus.

Ventral dorsal medial nucleus (*VDM*). This nucleus borders the ventral lateral nucleus at its dorsomedial surface. It shows a scattered, loose neuropil with mild SDA (E 46 ± 4), in which large cells are readily discernible by intense staining. There is a tendency to form cell groupings within the nucleus. The ventral dorsal medial nucleus extends dorsally between the lateral and the medial (dorsomedial) nucleus where it is continued by a strand of smaller cells with mild SDA. Ventromedially, cells of the central and paracentral nuclei continue the nucleus (Fig. 1E).

Ventral medial nucleus (*VM*). The ventral medial nucleus shows mild SDA (E 45 ± 5) distributed very homogeneously throughout its neuropil (Fig. 2 and 9).

Medial nuclear group

Parataenial nucleus (PT) and medial nucleus (dorsomedial nucleus; M). Both nuclei show mild SDA distributed homogeneously throughout the neuropil with no cell bodies discernible. Their medial boundaries with the midline nuclear group are indistinct. Laterally, the paratenial nucleus (E 47 ± 10) has sharp boundaries with the anterior nuclear group (Fig. 2); the medial nucleus (E 36 ± 7) (or the dorsomedial nucleus of many authors) is readily discerned from the lateral, ventral dorsal medial, and ventral lateral nucleus which all show stronger SDA.

Parafascicular nucleus (PF). These cells in the vicinity of the habenulo-peduncular tract show strong SDA in the perikarya and dendrites while the development of the reticular neuropil varies considerably among parts of the nucleus (Fig. 9).

Paracentral nucleus (PC). The paracentral nucleus is a region in which several patterns of adjacent regions blend with each other. A neuropil with a diffusely distributed, mild SDA blends medially from the midline nuclear group and also dorsally from the medial nucleus. Its structure becomes somewhat irregular in the paracentral nucleus where it is perforated by many small fibre bundles. Medium-sized nerve cells with mild SDA scatter laterally from the ventral dorsal medial and the lateral nuclei. The paracentral nucleus, thus, appears as a zone of transition rather than a well defined nucleus.

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Midline nuclear group (MI)

HESS discerned in the midline nuclear group (from dorsal to ventral): the paraventricular, central, rhomboid, and reuniens nuclei. Histochemically, the midline nuclear group forms one large complex of uniform structure and ill defined boundaries (Fig. 9). All its nuclei show weak or mild SDA (E 33 ± 5) distributed homogeneously throughout the neuropil. Only in the rhomboid and the central nuclei are cells with mild SDA discernible from the neuropil. These cells form a band which connects both ventral dorsal medial and paracentral nuclei across the midline; the band is thickened at the midline reflecting the shape of the rhomboid nucleus (Fig. 1E).

Posterior nucleus (P)

The posterior nucleus continues the lateral nucleus caudally showing an increase of SDA in the neuropil (E 77 ± 9), but no large neurons with strong SDA.

Lateral geniculate body (LG)

Its neuropil of very strong SDA (E 105 ± 13) is perforated by abundant, medium-sized, parallel fibre bundles which form a typical striped pattern (Fig. 5). There is also a slight inhomogeneity of the neuropil. Large nerve cells are barely discernible from the neuropil. The lateral geniculate body is subdivided into a large dorsal and a small ventral part by an intervening layer of white matter; there are no histochemical differences among these parts. A group of scattered, large nerve cells is discernible at the ventral pole of the nucleus, characterized by almost complete lack of neuropil and strong SDA in the cell bodies and dendrites.

Medial geniculate body (MG)

Its lateral part shows very strong SDA (E 105 ± 9) in the neuropil from which no cell bodies are discernible. The medial part shows a decrease of SDA in the neuropil

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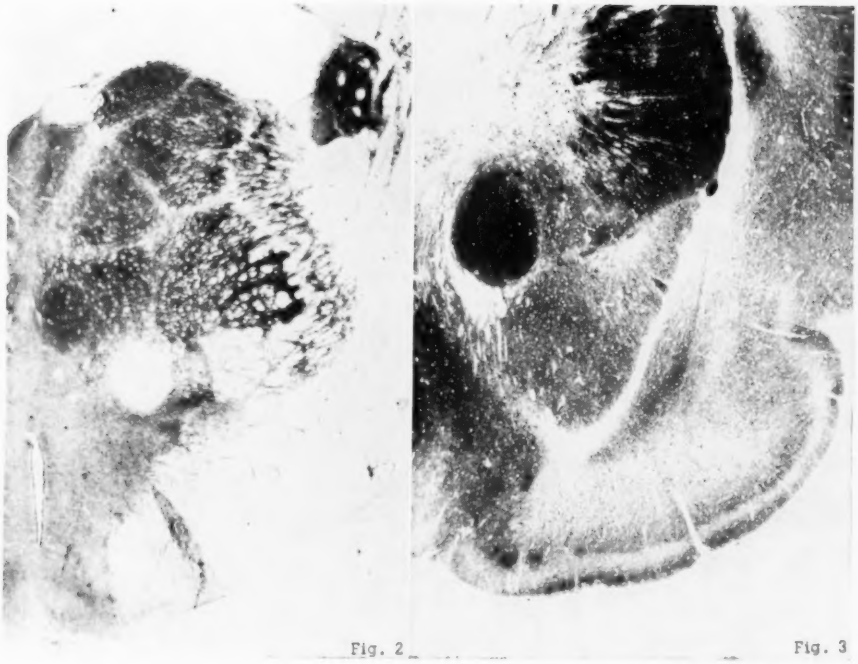

Fig. 2 Fig. 3

FIG. 2.—Rostral part of the thalamus; for identification of the nuclei compare Fig. 1D.

FIG. 3.—Nuclei of the amygdaloid complex; for identification see Fig. 1G.

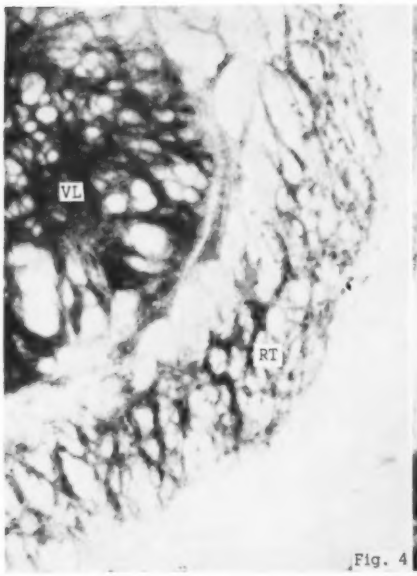

Fig. 4

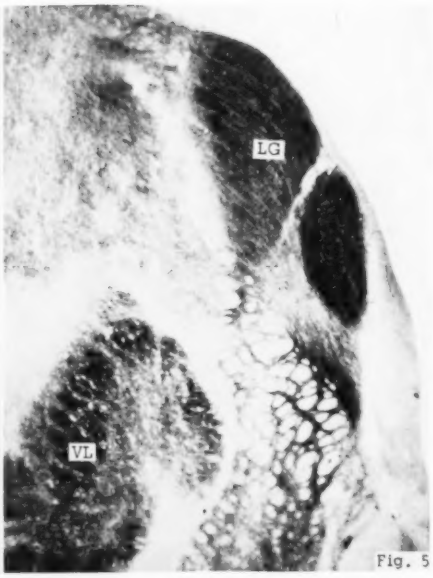

Fig. 5

FIG. 4.—Chemoarchitectonic pattern of the ventral lateral (VL) and the reticular (RT) thalamic nucleus.

FIG. 5.—Typical striped pattern of the lateral geniculate body (LG).

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where large, intensely stained nerve cells appear. Both parts blend with each other so that no sharp boundaries are discernible; a smaller cell group, however, is separated from the main nucleus medially, showing strong SDA in the cell bodies but almost none in the neuropil.

The dorsal part of the medial geniculate body shows mild SDA in its neuropil.

Pretectal area (*PR*)

The chemoarchitectonics of the pretectal area (E 71 ± 11) has been described in the preceding atlas of the midbrain of the guinea pig (FRIEDE, 1959).

Habenular nuclear group (Fig. 8)

The medial habenular nucleus (MH) shows a compact neuropil with very strong SDA; the caudal part of the nucleus shows a tendency to form clusters of extremely dense neuropil. The E 98 ± 11 is probably too low since the nucleus is small and light irradiation occurs at the margin of the section. Cell bodies generally are not discernible from the neuropil. The ependyma covering the nucleus is mostly in intimate contact with the neuropil but it may be separated from it by a narrow zone of weak SDA.

The lateral habenular nucleus (LH) shows strong SDA (E 71 ± 9) in its neuropil in which scattered, intensely stained nerve cells are visible. The arrangement of these cells is irregular, tending to form local aggregations.

Amygdaloid nuclei (Fig. 3)

If compared with the thalamus, the amygdaloid nuclei are characterized by weaker SDA and less accentuated gradations among individual nuclei. The nomenclature of the nuclei refers to JOHNSON (1957).

Central amygdaloid nucleus (*CE*). The medioventral part of the caudatum shows a decrease of SDA (E 61 ± 7) from which the oval-shaped central amygdaloid nucleus is sharply delineated. This nucleus is the most conspicuous one in the amygdaloid group, showing mild SDA (E 43 ± 5) distributed homogeneously throughout the neuropil without discernible cell bodies.

Lateral amygdaloid nucleus (*LA*). The dorsal pole of the lateral amygdaloid nucleus is wedge-shaped and interposed between the putamen and the claustrum; it is separated from the claustrum by the capsula externa. Its boundaries with the putamen and the central amygdaloid nucleus are defined by a distinct decrease of SDA. The ventral boundary with the basal amygdaloid nucleus is less distinct since mild SDA (E 35 ± 4) of the neuropil continues from one throughout the other. Cell bodies are discernible from the neuropil only in the large celled, basal part of the nucleus.

Basal amygdaloid nucleus (*B*). The basal amygdaloid nucleus (E 32 ± 6) lacks sharp boundaries with most of the neighbouring nuclei. Its appearance is similar to that of the lateral nucleus with which it merges dorsolaterally. Ventrally, the basal nucleus blends with the cortical amygdaloid nucleus.

Medial amygdaloid nucleus (*MA*). The medial amygdaloid nucleus shows slightly stronger SDA than the basal and the lateral nuclei; its boundaries are indistinct since the neuropil blends with any adjacent structure except the sharply delineated central nucleus. No cell bodies are discernible from the neuropil. If the medial amygdaloid nucleus is traced caudally, its medial pole is replaced by the stratum

moleculare of the hippocampus (Fig. 1G). Even the smallest portions of the latter are discernible by its very intense SDA (E 114).

Cortical amygdaloid nucleus (CO). The pyriforme cortex (area 51; for a detailed description see FRIEDE, 1960) shows a merging of the neuropil of its deep layers with that of the medial and the basal amygdaloid nucleus; the cortical amygdaloid nucleus (E 26 $\pm$ 5) occupies this area of transition and cannot be delineated sharply.

Massa intercalata. The intercalate cell groups of the amygdaloid complex are not discernible histochemically and blend with the neuropil of adjacent nuclei.

Anterior amygdaloid area. The anterior amygdaloid area represents the most rostal portion of the amygdaloid complex. It is an ill-defined zone of weak SDA distributed diffusely throughout the neuropil and resembling the lateral and basal amygdaloid nuclei. The neuropil lacks sharp boundaries with adjacent regions.

Nucleus of the lateral olfactory tract. This nucleus shows medium-sized cells with mild or strong SDA which are well discernible from a reticular neuropil.

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Septal nuclei

The description of the septal region (Fig. 1A, B) is difficult because of the frequency of local histochemical variations. The following provides an abbreviated account of the region; the nomenclature refers to JOHNSON (1957).

Medial line of septal gray. The *nucleus medialis septi* (SM). (E 37 $\pm$ 4) shows SDA in its neurons and their long dendrites but an almost complete absence of SDA in the neuropil.

A similar chemoarchitectonic pattern is found in the *nucleus of the diagonal band of Broca* (NDB) which sweeps laterally from the medial septal nucleus. The nucleus of the diagonal band, however, shows an irregular neuropil with mild SDA (E 48 $\pm$ 5).

The *bed nuclei of the anterior commissure* (BED) show a neuropil with diffusely distributed, mild SDA in which no cell bodies are discernible.

The *nucleus triangularis* (T) is conspicuous by strong SDA in its irregular neuropil and small, intensely stained cells.

Lateral line of septal nuclei. The *nucleus lateralis septi* (SL) shows a gradation of strong, diffusely distributed SDA in its ventral part and weak SDA with predominating cell bodies in its dorsal part (transition zone: E 55 $\pm$ 5).

The chemoarchitectonics of the *nucl. accumbens* (AC) is identical with that of the nucl. caudatus with which its neuropil is continuous around the frontal horn of the lateral ventricle (Fig. 9). The nucl. accumbens is discernible from the lateral septal nuclei by stronger SDA.

The *nucleus septalis fimbrials* (SF) resembles closely the medial septal nucleus (E 37 $\pm$ 4).

Corpus mammillare

The nucl. mammillaris medialis (MAM M) shows very strong SDA (E 103 $\pm$ 12) distributed homogeneously in its neuropil. The neuropil of the nucl. mammillaris lateralis (MAM L) has a looser structure and shows very intensely stained neurons (E 59 $\pm$ 9). The neuropil of both nuclei is separated from the surface of the brain by a narrow layer of weak SDA.

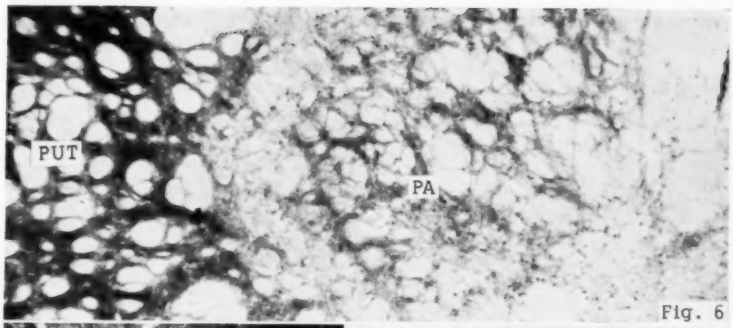

Fig. 6

Fig. 7

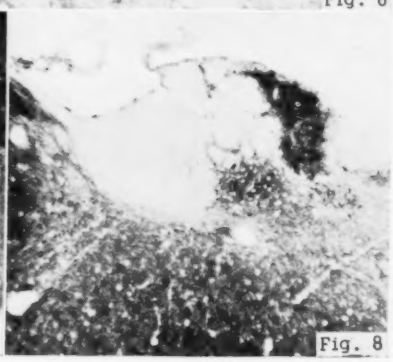

Fig. 8

FIG. 6.—Boundary of putamen and pallidum as characterized by a sharp decrease of enzymic activity in the latter.

FIG. 7.—Medial (right) and lateral (below and left) habenular nuclei.

FIG. 8.—Absence of enzymic activity in the ependyma lining the recessus infundibuli.

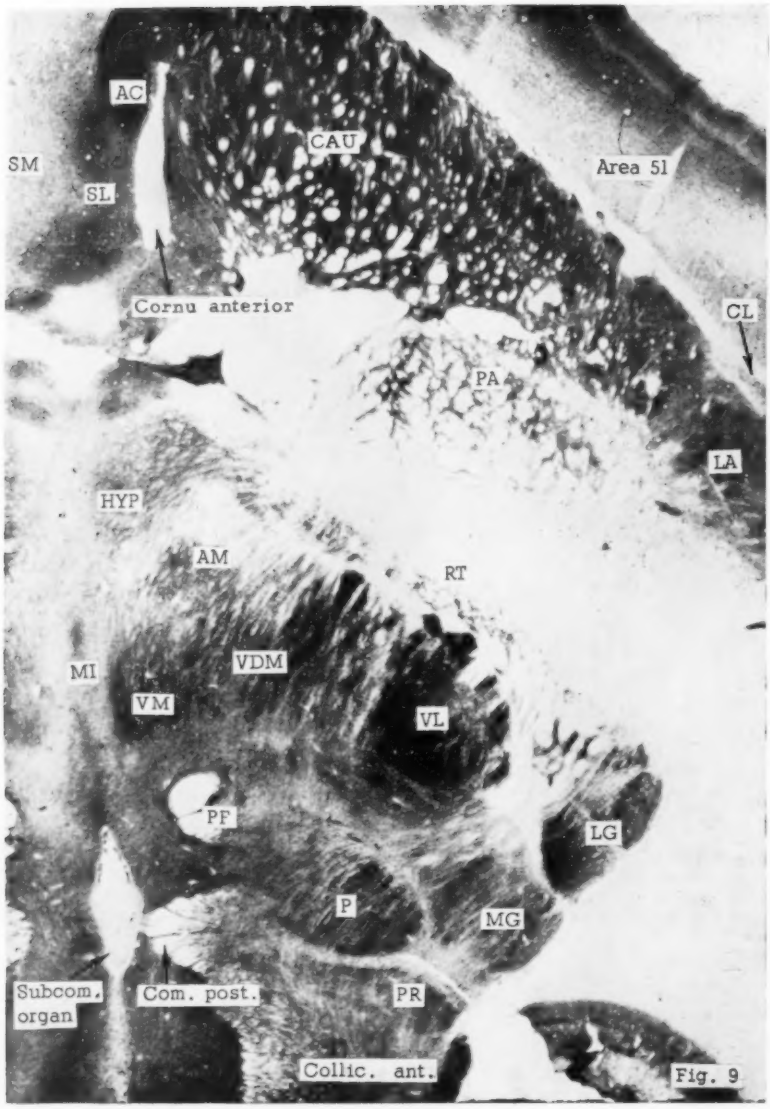

FIG. 9.—Section cut fronto-occipitally and about parallel to the basis of the skull; the majority of the nuclei described in this article are seen and can be compared conveniently.

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Corpus subthalamicum (*SUB*)

The corpus subthalamicum (E 81 ± 9) is discernible from the substantia nigra (E 29 ± 4) by stronger SDA; the neuropil has slightly irregular structure in which darkly stained nerve cells are discernible.

Zona incerta (*ZI*)

The zona incerta shows a neuropil of mild SDA (E 38 ± 5) arranged in loose strands between the fibre bundles; few cell bodies are discernible. The neuropil of the zona incerta blends dorsally with the nucl. reticularis thalami but has a more homogeneous structure than the latter.

Hypothalamus (*HYP*)

The histochemical appearance of the hypothalamus resembles that of the central gray substance, showing weak SDA (E 24 ± 3) distributed homogeneously throughout the neuropil (Fig. 2). A few nerve cells are discernible from the neuropil by slightly stronger SDA. There is no evident histochemical subdivision of the hypothalamus except that SDA prevails in perikarya in the lateral parts and prevails in the neuropil in the medial part. The nuclei supraoptici and paraventriculares are discernible by mild SDA in their cells but the enzymic activity does not largely exceed that of the surrounding tissue.

The ependyma lining the recessus infudibuli lacks SDA in contrast to all other parts of the ventricular system (Fig. 7).

DISCUSSION

Several aspects of the histochemical distribution of succinic dehydrogenase (relations to vascularization, cell statistics, etc.) were dealt with in the preceding articles of this series and will not be touched upon here. The chemoarchitectonic subdivision of the thalamus stimulated interest in its possible correlation with functional anatomy. The comprehensive text of KAPPERS, HUBER and CROSBY (1936) may be consulted as a reference for cortico-thalamic connections. A comparison of measurements shows a histochemical similarity of thalamic nuclei and their cortical projection areas (Fig. 10).

Differences among neocortical areas are established mainly by gradations of enzymic activity in the neuropil of the third and fourth layers in which the thalamic radiations terminate. All the primary sensory areas are characterized by very strong SDA in their upper cellular layers (postcentral cortex, E 104; auditory cortex, E 97; and visual cortex, E 93); likewise very strong SDA is found in the thalamic projection nuclei (ventral lateral nucleus, E 118; medial geniculate body, E 105; lateral geniculate body, E 105). It may be more than a coincidence that both the postcentral region (area 1-3-5) and the ventrolateral thalamic nucleus show an exceptionally irregular structure of the neuropil: the postcentral cortex exhibits a segmentation of the third layer, which is characteristic for this area, and the ventral thalamic nucleus shows a tendency to form clusters of very compact neuropil which is not observed to a similar extent in other thalamic nuclei. A similarly irregular neuropil is also found in the nuclei of the dorsal tracts. The enzymic activity in the upper layers of the parietal cortex is weaker than that of the sensory areas (E 60); likewise, the lateral thalamic nucleus shows weaker SDA (E 52). The upper layers of the frontal cortex

exhibit mild SDA (E 50) with a gradual decrease towards the frontal pole; mild SDA is also found in the medial (or dorsomedial) thalamic nucleus (E 36) and the anterior part of the ventrolateral nucleus. Enzymic activity is weakest in the thalamic midline nuclear group (E 33) which has anatomical and functional relations to the hypothalamus which also shows weak SDA (E 24). Of interest is the prevalence of enzymic activity either in the neuropil or in the perikarya. Such gradations establish the pattern of an inner and outer nuclear group and an inner and outer lamina which agrees with the concept of the functional organization of the thalamus in higher mammals and man.

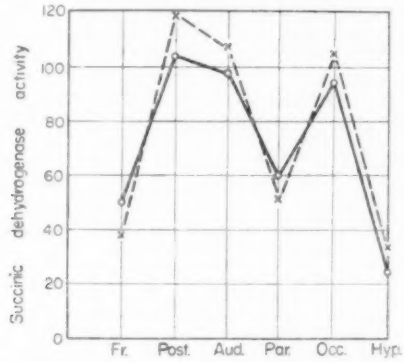

FIG. 10.—Comparison of enzymic activity in projection fields (○) and related thalamic nuclei (×): Fr.: frontal cortex and dorsomedial nucleus; Post.: postcentral cortex and ventral lateral nucleus; Aud.: auditory cortex and medial geniculate body; Par.: parietal cortex and lateral nucleus; Occ.: visual cortex and lateral geniculate body; Hyp.: hypothalmus and midline nuclear group.

There is a general support for the concept that the cytological pattern of enzymic activity reflects the functional properties of a nucleus: prevalence of enzymic activity in the perikarya over that in the neuropil is indicative of an 'effector' function; whereas prevalence of enzymic activity in the neuropil over that in the perikarya indicates a receptive, associative, or integrative function. This principle is found in the cerebral cortex, the organization of the thalamus, the nucl. lentiformis with its striking dissimilarity of putamen and pallidum, and many nuclei of the brain stem. The histochemical patterns described herein and in the preceding articles, therefore, not only provide a chemical topography of the brain but may serve as well as a key to the functional organization of gray matter.

SUMMARY

The histochemical distribution of succinic dehydrogenase activity in the diencephalon and certain basal telencephalic centres of the guinea pig is described and measured photometrically, thus completing a detailed mapping of this enzyme in the entire brain of the guinea pig.

The thalamic nuclei show a rich chemoarchitectonic subdivision: strongest activity is found in the anterior-dorsal, ventro-lateral, habenular, medial, and lateral geniculate nuclei. Weaker activity is found in the lateral, posterior, and medial nuclei and weakest activity in the midline nuclear group. The amygdaloid nuclei differ little from each other and are generally characterized by weaker enzymic activity than is found in the thalamic nuclei.

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A comparison of measurements in thalamic nuclei and cortical areas shows that the enzymic activity in individual thalamic nuclei is of the same order as that in their cortical projection areas.

ABBREVIATIONS

A5l	area pyriformis
AC	nucleus accumbens
AD	anterior dorsal nucleus
AM	anterior medial nucleus
AV	anterior ventral nucleus
B	basal amygdaloid nucleus
BED	bed nucleus of the anterior commissure
C	central nucleus
CAU	caudate nucleus
CE	central amygdaloid nucleus
COM A	commissura anterior
CL	claustrum
CO	cortical amygdaloid nucleus
DARK	nucleus of Darkschewitsch
GR C	griseum centrale
H	hippocampus
HYP	hypothalamus
INT	nucleus interstitialis of Cajal
IP	interpeduncular nucleus
L	lateral nucleus
LA	lateral amygdaloid nucleus
LG	lateral geniculate body
LH	lateral habenular nucleus
M	medial nucleus (or dorsomedial nucleus)
MA	medial amygdaloid nucleus
MAM M	medial mammillar nucleus
MAM L	lateral mammillar nucleus
MG	medial geniculate body
MH	medial habenular nucleus
MI	midline nuclear group
NDB	nucleus of the diagonal band of Broca
NIG	nucleus niger
OPT	optic tract
P	posterior nucleus
PA	nucleus pallidus
PC	paracentral nucleus
PF	parafascicular nucleus
PR	pretectal area
PT	parataenial nucleus
RT	reticular nucleus
SF	fimbrial septal nucleus
SL	lateral septal nucleus
SM	medial septal nucleus (in the thalamus also used for stria medullaris thalami)
SUB	corpus subthalamicus
T	nucleus triangularis
VDM	ventral dorsal medial nucleus
VL	ventral lateral nucleus
VM	ventral medial nucleus
ZI	zona incerta

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CYTOCHEMICAL LOCALIZATION OF THE OXIDATIVE ENZYME SYSTEMS IN THE RETINA—I

DIAPHORASES AND DEHYDROGENASES

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SOME attempts have been made to correlate the fine structure of the retina with basic biochemical mechanisms. Succinic dehydrogenase has been investigated by FRANCIS (1952) and WISLOCKI and SIDMAN (1954), and more recently a series of articles have been published (KUWABARA *et al.*, 1959; KUWABARA and COGAN, 1959; COGAN and KUWABARA; 1959), in which tetrazolium studies on the retina are described. These and other studies have suggested that there may be functional counterparts to the submicroscopic morphology. Moreover, it has been stated that there are two different types of dehydrogenase localized in the retina, one represented by the succinic dehydrogenase system and the other by the DPN-linked lactic dehydrogenase.

In the present communication results are given of cytochemical studies on the distribution of various oxidative enzymes including succinic and lactic dehydrogenases in the retina of different animal species. For the demonstration of the activity of the diaphorases and dehydrogenases the newer techniques have been used which make it possible to localize the enzyme activity at a cytological, i.e. mitochondrial level (PEARSE and SCARPELLI, 1959).

MATERIAL AND METHODS

Healthy untreated frogs (Rana temporaria), mice, rats, guinea pigs and rabbits were used. The animals were killed by a blow on the neck and the eyes were immediately removed and frozen. Sections were cut with a cold microtome (cryostat) at 15 μ. Whole eyes were usually sectioned, but on a few occasions the lens and the vitreous body were taken away before cutting. Some frogs were adapted to dark by keeping them in a dark room for 16 hr. The killing of dark-adapted frogs and the subsequent dissecting and freezing of their eyes took place in dark red light.

Sections mounted on coverslips were incubated for the demonstration of diphosphopyridine and triphosphopyridine nucleotide diaphorases (DPND and TPND) and of succinic (SD), lactic, glucose-6-phosphate, β-hydroxybutyrate, alcohol and α-glycerophosphate dehydrogenases. The methods of HESS *et al.* (1958) and SCARPELLI *et al.* (1958) were followed; the final electron acceptor was 2-(4:5-dimethylthiazol-2-yl)3:5-diphenyl tetrazolium bromide (MTT) in the case of diaphorases and succinic, lactic, glucose-6-phosphate and β-hydroxybutyrate dehydrogenases. For the visualization of the remaining dehydrogenases Nitro-BT was used.

Sometimes eyes were fixed in cold formol–calcium overnight and both DPND and lactic dehydrogenase were demonstrated in frozen sections of fixed tissue as well (NOVIKOFF and MASEK, 1958).

RESULTS

The results of the distribution of the diaphorases and succinic dehydrogenase are summarized in Table 1. Table 2 shows the mean distribution of the enzymes in the retinas of the species used.

TABLE 1.—SITES OF ACTIVITY OF SUCCINIC DEHYDROGENASE (SD) AND PHOSPHOPYRIDINE NUCLEOTIDE DIAPHORASES (DPND AND TPND)

Species	Layer																				
	Ganglion cell			Inner plex.			Inner nucl.			Outer plex.			Outer nucl.			Rods and cones Inner segment			Outer segment		
	SD	DPND	TPND	SD	DPND	TPND	SD	DPND	TPND	SD	DPND	TPND	SD	DPND	TPND	SD	DPND	TPND	SD	DPND	TPND
Frog	0	0	0	1	0	1	0	0	1	2	1	3	1	0	1	3	2	2	0	0	0
Mouse	0	3	4	1	2	2	0	1	2	2	3	4	0	0	0	4	4	3	0	0	0
Rat	0	2	2	1	2	2	0	1	1	4	2	3	0	0	0	4	3	3	0	0	0
Guinea pig	0	4	3	0	1	2	0	1	2	0	1	2	0	1	2	4	3	0	0	0	0
Rabbit	0	4	3	0	0	0	0	0	0	0	1	1	0	0	0	4	2	2	0	0	0

TABLE 2.—THE MEAN DISTRIBUTION OF SOME OXIDATIVE ENZYMES IN THE RETINAL LAYERS OF THE FIVE SPECIES STUDIED

Succinic dehydrogenase	Layer	Diaphorases		Dehydrogenases				
		DPND	TPND	lactic	β-hydroxy-butyric	glucose-6-phosphate	α-glycero-phosphate	alcohol
0	Ganglion cell	3	3	4	3	3	3	3
1	Inner plexiform	2	2	2	2	2	2	2
0	Inner nuclear	1	1	1	2	0	0	1
3	Outer plexiform	2	3	3	2	2	2	2
0	Outer nuclear	0	0	0	0	0	0	0
4	Rods and cones — Inner segment	3	3	4	1	1	2	2
0	Rods and cones — Outer segment	0	0	0	0	0	0	0

Succinic dehydrogenase

This enzyme exhibited a strong activity in the inner segment of the rods and cones (Figs. 2 and 6). A large number of formazan granules were concentrated in the region of the ellipsoids in all the species. A moderate activity was seen in some species in the outer plexiform layer, where the mitochondrial formazan deposits were most numerous on the outer side of this layer. Activity in this layer was most marked in the rat and mouse retina (Fig. 2). A weak diffuse staining was infrequently observed in the inner plexiform layer, as well. While this was the general outlook of SD activity, the retina of the rabbit (Fig. 6) and the guinea pig exhibited a different pattern of SD activity; the enzyme was localized in these species in the ellipsoid region only, all the other parts of the retina always remaining unstained. The ganglion cells were negative in all species.

DPN- and TPN-diaphorases

A strong granular formazan deposit was found localized in all species in the inner segment of the rods and cones, when sections were incubated using reduced DPN or TPN as substrates (Figs. 1, 3, 5, 7, 9 and 10). In the retina of dark-adapted frogs the reaction product could be seen precisely as 10–20 formazan dots per cell, which were localized to the ellipsoids of the photo-receptor cells (Fig. 7). Besides the ellipsoid region, the ganglion cell layer exhibited strong diaphorase activity in all species except the frog, whose ganglion cells did not give any reaction. In the retina of the guinea pig and the rabbit the ganglion cells were clearly more active than the visual cells. The inner plexiform and nuclear layers were usually stained as well. In the guinea pig particularly, sharp, deeply stained fibres were observed, which run from the ganglion cell layer down to the outer nuclear layer (Figs. 9 and 10). Smaller diaphorase-positive fibres were usually found in the mouse and rabbit as well.

There were hardly any differences between the two diaphorases. However, it could be noticed that TPND was often relatively more active in the ganglion cells than in the ellipsoids.

DPN- and TPN-linked dehydrogenases

All the specific dehydrogenases broadly followed the localization of the corresponding diaphorase. Thus, they exhibited two sites of maximal activity: in the ganglion cell layer and in the inner segments of the rods and cones. Naturally, where the diaphorase was lacking, as in the ganglion cells of the frog retina, no co-enzyme dependent dehydrogenase activity could be demonstrated. A few comments on the variation between species and substrates are, however, made.

Lactic dehydrogenase. An intense staining of the ellipsoid region was noted in all species (Fig. 4). The ganglion cell layer also showed intense LD activity except for the frog eye. Some staining of both plexiform layers could be seen; in the mouse retina this was clearly localized in the Müller's fibres, in the other species the staining had a more diffuse look. Cyanide could not be shown to have any inhibitory effect; on the contrary, its withdrawal resulted only in a general decrease of the reaction. Fixation overnight in cold formalin did not destroy this enzyme but the incubation time was longer with fixed sections than with fresh ones.

Glucose-6-phosphate dehydrogenase. The highest activity of this TPN-linked enzyme was found in the ganglion cells. Some staining of the photo-receptor cell layer

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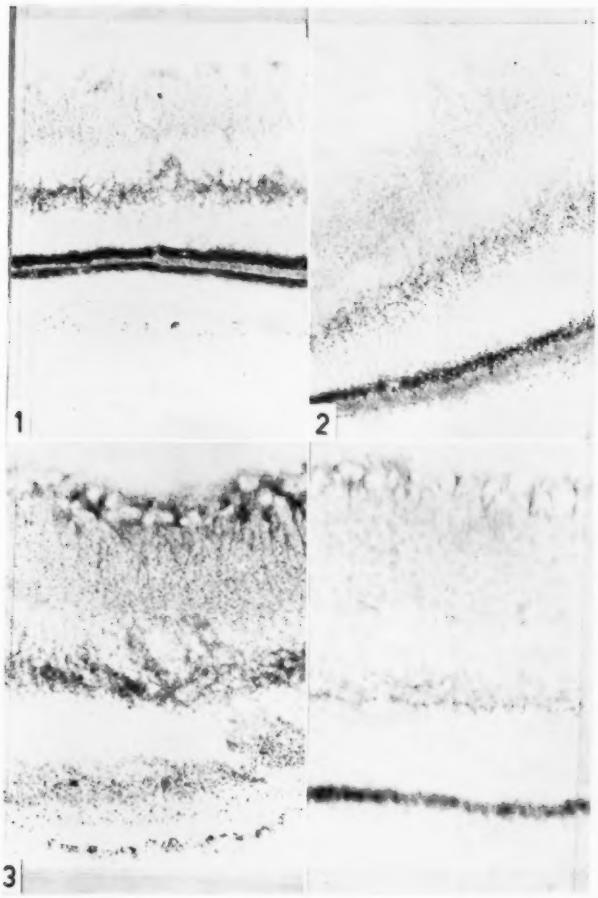

FIG. 1.—DPN-diaphorase activity in the retina of the mouse. The ellipsoids are very intensely stained; a moderate enzyme activity is also seen in the plexiform layers and in the ganglion cells. ×192.

FIG. 2.—Succinic dehydrogenase in the mouse retina. The granular (mitochondrial) form of the precipitate is well visible. The inner segments of the rods and cones are most active, but the outer plexiform layer also shows moderate activity. ×246.

FIG. 3.—Mouse retina, TPN-diaphorase. In the inner plexiform layer some vertical fibres are visible. ×246.

FIG. 4.—Mouse retina, lactic dehydrogenase–cobalt–MTT method. The ellipsoids show the most intense activity. Note the formazan granules on the outer side of the outer plexiform layer probably representing the rod and cone outer spherules. ×246.

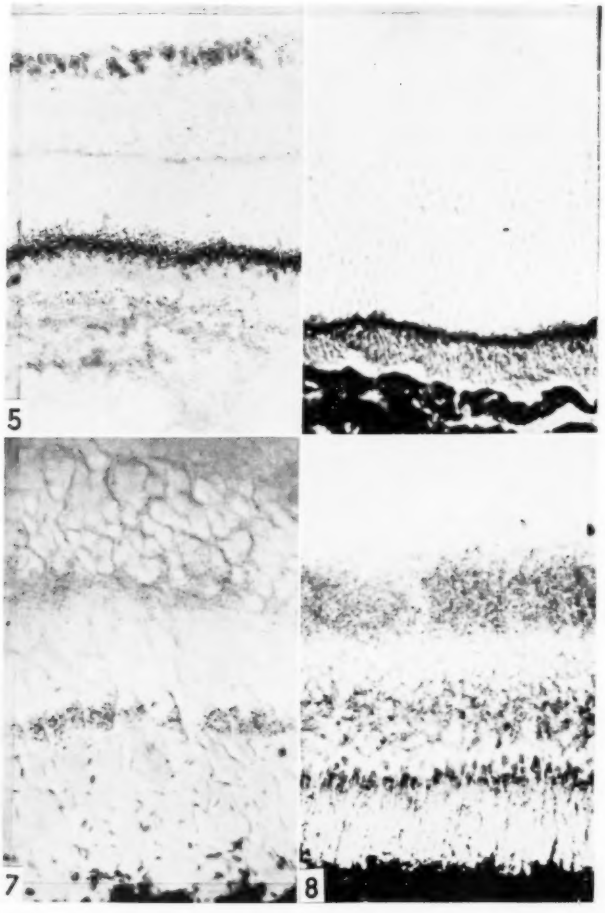

FIG. 5.—Rabbit retina, DPN-diaphorase. The ellipsoids and the ganglion cells show most intense enzyme activity, but even the outer plexiform layer is clearly stained. ×228.

FIG. 6.—Rabbit retina; succinic dehydrogenase activity is localized to the inner segments of the visual cells only. ×300.

FIG. 7.—DPND reaction in the frog's retina. The intramitochondrial formazan granules are precisely located to the ellipsoids, each of them incorporating some 10 to 20 granules. ×480.

FIG. 8.—Same retina, alcohol dehydrogenase, dark adapted animal. Enzyme activity present in the inner segments of the visual cells and in both plexiform layers. ×192.

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was also seen; in the plexiform layers the staining was very weak, but in most instances clearly localized in the Müller's fibres.

β-Hydroxybutyrate dehydrogenase. No, or very weak, activity could be found in the cones and rods. The ganglion cell layer showed moderate staining, as did the fibres in the plexiform layers and in the inner nuclear layer.

Alcohol dehydrogenase. This enzyme showed a maximal activity in the ganglion cell layer, but the ellipsoids also were stained in all species (Figs. 8 and 11). Even the plexiform layers exhibited alcohol dehydrogenase activity. In the guinea pig, particularly, this enzyme was concentrated on thick fibres running through the retina (Fig. 11). The fibres were especially well seen in the outer plexiform and inner nuclear layers. In the ganglion cell layer a thickening of the stained fibres was often observed.

α-Glycerophosphate dehydrogenase. This enzyme gave a relatively weak reaction in all species. The ganglion cell layer was again the most active, but even the ellipsoids showed some activity in most species, as did the plexiforms (Fig. 12).

DISCUSSION

The present study confirms by cytochemical methods the observations made with the electron microscope on the distribution of mitochondria in the photo-receptor cells of the retina. The intramitochondrial formazan deposits were found to be concentrated in most instances in the inner segment of the rods and cones, and another layer of precipitate was seen in the outer plexiform layer, mostly near its outer surface; the electron microscope studies have revealed numerous mitochondria in the same sites (SJÖSTRAND, 1953; DE ROBERTIS, 1956; LADMAN, 1958). No electron microscope studies have, however, been made on the submicroscopic cytology of the Müller's cells and the ganglion cells of the retina, although the structure of the retinal synapses have been described in detail (SJÖSTRAND, 1958). In the experiments reported here, aggregations of typical formazan dots were found in the ganglion cells of the retina, when diaphorases or pyridine nucleotide-linked dehydrogenases were demonstrated. Moreover, the granular reaction product found in the Müller's fibres suggests that the cytoplasm of these cells contains mitochondria.

LOWRY, ROBERTS and LEWIS (1956) were the first to observe a clear difference in the oxidative metabolic pathways between the inner segment of the rods and cones and the other inner layers of the retina. They studied malic and lactic dehydrogenases microchemically and found an inverse correlation between their activities, the highest malic dehydrogenase level being found in the inner segments of the rods and cones. In the present study succinic dehydrogenase, representing the citric acid cycle, was also found to be concentrated to the same region. Similar localization has been noticed also by WISLOCKI and SIDMAN (1954) and by others. It may therefore be concluded that the aggregates of mitochondria filling the apical portion of the inner segments of the rods and cones is an obvious locus of those dehydrogenases which take part in the tricarboxylic acid cycle.

On the other hand, our results indicate that the maximal activity of DPN-diaphorase and various DPN- and TPN-linked dehydrogenases is in the inner layers of the retina, particularly in the layer of ganglion cells. LOWRY *et al.* (1956) pointed out that the oxidative metabolism of the inner layers of the retina is characterized by a high rate of glycolysis; this was based on their observation that the lactic dehydrogenase level was low in the photo-receptor cell layer but high in the inner layers, especially in

the outer plexiform, inner nuclear and ganglion cell layers. Moreover, they found differences between rabbit and monkey retinas, and concluded that glycolytic metabolism is active where the second set of choroid vessels is missing.

Our studies included dehydrogenases involved both in the early phases of glycolysis (α-glycerophosphate dehydrogenase) and in its later reactions (alcohol and lactic dehydrogenases). All these enzymes exhibited peak activity in the inner retinal layers, especially in the ganglion cell layer. A positive reaction was, however, noticed in other layers as well, particularly in the inner segment of the photoreceptor cells.

So far the most comprehensive histochemical study on retinal oxidative enzymes has been made by KUWABARA and COGAN (1959). The techniques used by these authors differ, however, in many respects from those used in the present work. First, they incubated pieces of retina in the substrate media, with subsequent embedding in gelatin and sectioning in the frozen state. Secondly, they used blue tetrazolium as the electron acceptor. When relatively large tissue blocks are incubated, the time of incubation must be long to ensure a reasonable penetration of the substrate and the salt into the various tissue layers. The long incubation time makes it, on the other hand, impossible to use tetrazolium salts of high redox potential and, hence, of improved sensitivity.

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In the present work, fresh-frozen cold microtome sections were used. They were entirely satisfactory as far as the preservation of the cytological structure was concerned. Moreover, they made possible the use of the newer tetrazolium salts of high redox potential. As pointed out by PEARSE (1960), only these salts (Nitro-BT and MTT) can be expected to give a precise cytological localization of the tetrazolium reducing enzyme systems, the older salts being satisfactory for localization at a histological level only. In our present findings, the strict correlation between cytochemically demonstrable mitochondria and those described in electron microscopy is a further support to this view.

A further difficulty in comparing many earlier tetrazolium studies with the present one, is the two-coloured reaction product obtained with the ditetrazoles commonly used. Most authors seem to prefer the view that both red and blue precipitates are indicative of different degrees of enzyme activity. When a monotetrazole like MTT is used the result is, however, more clear-cut and does not leave as much space for speculation.

In spite of many technical differences our findings generally agree with those of KUWABARA and COGAN. Our results support the view of these authors that the succinic dehydrogenase system is to a great part localized in the ellipsoid part of the rods and cones. In some of our species, however, succinic dehydrogenase activity could also be seen in the plexiform layers. The other dehydrogenases included in our study were, on the contrary, usually most active in the ganglion cell layer. They all exhibited, however, some activity even at the level of the ellipsoids.

Concerning the distribution of the diaphorases, our present findings differ considerably from those of KUWABARA and COGAN. Our observations strongly suggest that these enzymes are distributed more evenly throughout the whole retina; in many species, indeed, the activity was as strong in the ellipsoids as in the ganglion cell layer, and in the plexiforms a clear positive reaction was found.

The pyridine nucleotide-linked dehydrogenases were mostly concentrated in the ganglion cell layer but definite activity was observed in the outer layers and in the

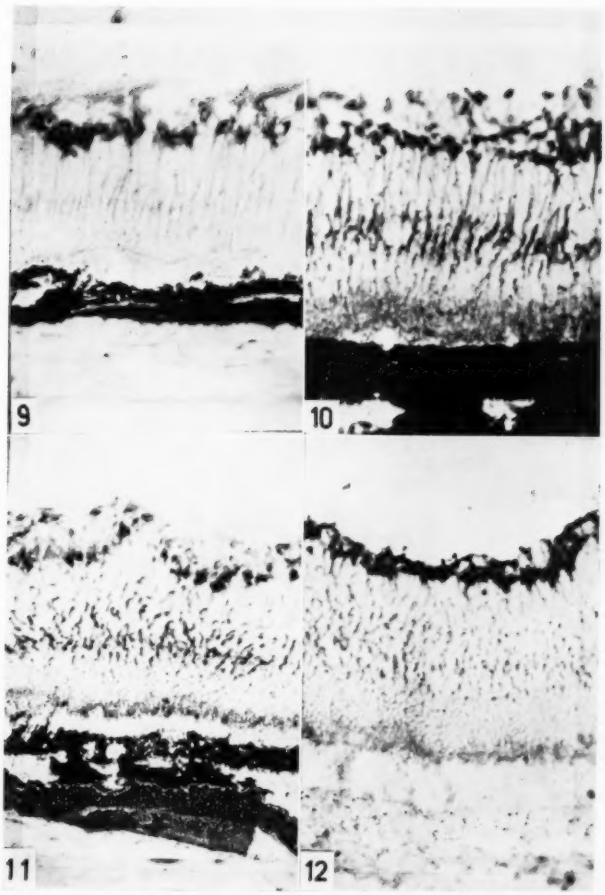

FIG. 9.—Guinea pig retina, DPN-diaphorase. The ganglion cells are by far the most active, fibres well visible. ×180.

FIG. 10.—Same retina, TPN-diaphorase. Thick vertical fibres can be seen to run from the ganglion cells down to the photoreceptors. ×180.

FIG. 11.—Alcohol dehydrogenase in the retina of the guinea pig. Besides the ganglion cell layer and the layer of ellipsoids, the Müller's fibres show intense enzyme activity. ×180.

FIG. 12.—Guinea pig retina, α-glycerophosphate dehydrogenase. The ganglion cells are clearly most intensely stained. ×180.

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ellipsoids. Hence, although the glycolytic enzymes are more active in the inner retinal layers, their existence in other layers suggests a more mixed and complicated oxidative metabolic pattern for the retina than simply a division into an aerobic region and an anaerobic one.

SUMMARY

The distribution of pyridine nucleotide diaphorases, succinic dehydrogenase and five pyridine nucleotide-linked dehydrogenases in the retina of five animal species have been studied. Newer tetrazolium salts of high redox potential were used, yielding a precise cytological, even mitochondrial, localization of the enzymes.

Both diaphorases were relatively uniformly distributed, the strongest activity being in the ellipsoid region of the rods and cones and in the ganglion cells, but the plexiform layers exhibited definite enzyme activity also. Succinic dehydrogenase was either entirely or very largely situated in the ellipsoids and in the outer side of the outer plexiform layer, a localization corresponding to the distribution of mitochondria in the retina as seen with the electron microscope. Those of the various pyridine nucleotide-linked dehydrogenases which are particularly involved in glycolysis showed maximal activity in the ganglion cell layer, but activity was also present in other layers.

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CYTOCHEMICAL LOCALIZATION OF THE OXIDATIVE ENZYME SYSTEMS IN THE RETINA—II

CYTOCHROME OXIDASE

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IN THEIR monograph on the biochemistry of the eye, PIRIE and VAN HEYNINGEN (1956) wrote: "It is more important to know the distribution of substances within the retina than to know the total in the whole tissue . . . ." In another connection, the authors pointed out that cytochrome oxidase is abundant in retina, but its distribution is described loosely by referring it as being present largely in the cellular layer. Using a biochemical approach EICHEL and SWANSON (1956) have demonstrated that normal rabbit retina possesses relatively low cytochrome oxidase activity, but they did not study the sites of this activity within the retina. In article I of this series (NIEMI and MERENMIES, 1960) the localization of diaphorases and various dehydrogenases in the retina was reported. It was pointed out, as has been done by others (FRANCIS, 1952; WISLOCKI and SIDMAN, 1954; COGAN and KUWABARA, 1959) that succinic dehydrogenase is in most instances located only in those parts of the photo-receptor cells in which mitochondria have been observed by electron microscopy, i.e. in the ellipsoids and in the outer spherules of the rods.

Since succinic dehydrogenase is a part of the succinic oxidase system of KEILIN and HARTREE (1938), it seemed of interest to investigate cytochemically the other part of the system also, i.e. cytochrome oxidase, in the retina. In the present paper results will be given of such studies, in which a recent modification by BURSTONE (1959) of the original 'Nadi' reaction was used for the visualization of the tissue oxidase activity.

MATERIAL AND METHODS

Eyes of the frog (Rana temporaria), the mouse, the rat, the guinea pig and the rabbit were used. They were rapidly frozen after removal: fresh-frozen sections were cut at 20 μ in a cold microtome. They were allowed to dry at room temperature for a few minutes and subsequently placed in the incubating mixture. This was prepared according to BURSTONE (1959); N-phenyl-*p*-phenylenediamine·2HCl was used as the amine and the coupler used was 4-chloro-5-methyl-0-acetanisidine. Exogenous cytochrome *c* was usually included in the incubation solution in a concentration of 1 mg/100 ml. The enzyme was inhibited by adding 10^{-3} M-KCN to the incubation mixture. On a few occasions, 7·5% polyvinylpyrrolidone was added to the incubation solution for the osmotic protection of mitochondria (SCARPELLI and PEARSE, 1958).

Sudan III and IV were used for the demonstration of lipids. For the extraction of lipid substances, the sections were treated in ice-cold acetone for 10 min.

Rat liver homogenate was used for the diffusion experiments. Liver pieces were homogenized in an equal volume of distilled water and centrifuged at 3000 rev/min for 20 min; 2–3 ml of the supernatant fluid were added to 10 ml of fresh reaction mixture. Heat-inactivated sections were incubated in this solution with continuous agitation.

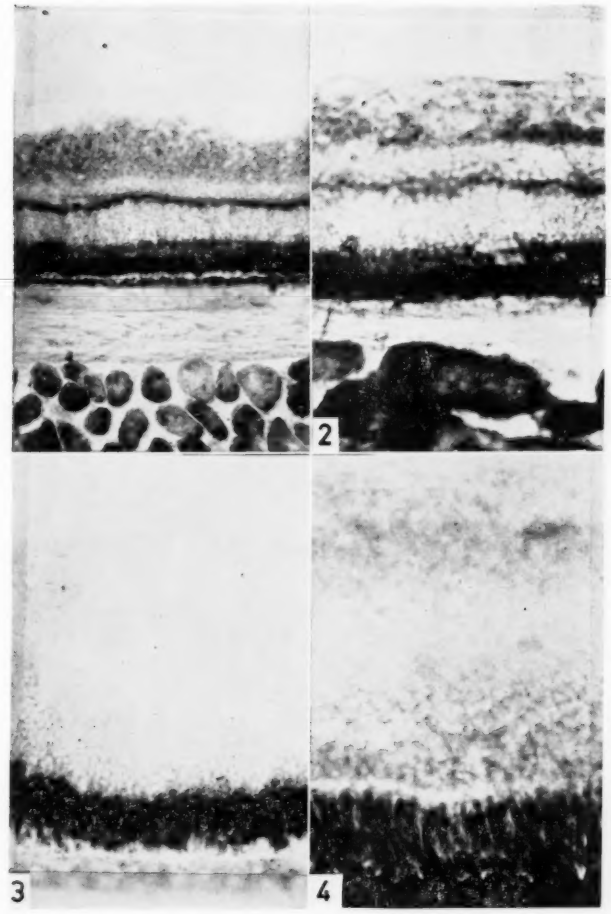

FIG. 1.—Cytochrome oxidase activity in the retina of the rat. The outer limbs of the rods and cones are very intensely stained. The ellipsoid region and the outer plexiform layer show definite enzyme activity; the staining of the inner plexiform layer is weaker. ×79.

FIG. 2.—Same reaction in the retina of the mouse; the enzyme distribution corresponds to that in the rat retina. ×198.

FIG. 3.—The retina of the guinea pig. Cytochrome oxidase is present only in the rods, the outer limbs exhibiting more intense staining; the granular form of the precipitate is well visible. ×222.

FIG. 4.—Frog's retina. Besides the photoreceptors, the plexiform layers show some staining. ×234.

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RESULTS

Distinct staining of various retinal components was produced after about 30 min incubation; however, in the case of the frog 2 hours were necessary for a good reaction. The addition of cytochrome *c* reduced the incubation time, which without it was as long as 4–5 hr. This time lapse caused considerable disruption of the tissue elements. The addition of polyvinylpyrrolidone to the incubation mixture made an even longer incubation time necessary, and did not improve the cytological picture. Addition of KCN in a concentration of 10^{-3} M inhibited the reaction completely.

The staining of the retinas of the various species is illustrated in Figs. 1–4. As can be seen, the most intense staining is situated in all species in the outer limb of the photo-receptor cells. While the staining of this part was generally diffuse, a finely granular precipitate was usually observed in the inner segment of the photosensitive cells of all the species studied. In the retina of the mouse and the rat (Figs. 1 and 2), the outer plexiform layer also exhibited marked oxidase activity, but in the frog (Fig. 4) this region was stained only slightly and in the guinea pig (Fig. 3) and rabbit not at all. In the inner plexiform as well as in the ganglion cell layers of the mouse and the rat a positive reaction was noticed, but in the other three species only faint (the frog) staining was found or none at all.

The dye produced by the rat liver homogenate did not diffuse to any of the retinal layers. Pretreatment of the sections with acetone did not change the results at all.

DISCUSSION

In all the animals studied the outer segment of the photo-receptor cells was the site of maximal precipitation of the azomethine dyestuff. In the first instance, this finding may be surprising. The outer segment of the rods and cones is generally accepted to be a passive site of storage for visual pigment rather than a metabolically active structure. This view is mainly based on the submicroscopic organization of the outer segments, which consist of a simple pile of numerous flattened sacs (De Robertis and Lasansky, 1958). Also some earlier findings (Schmidt, 1951) are in favour of the view that the outer limb has a specialized photo-receptor function only and its metabolic functions depend on supply from the inner segment where most of the general cytoplasmic components of vegetative cells are present. Thus, numerous mitochondria have been observed by electron microscopy (De Robertis, 1956) and a high activity of many dehydrogenases has been demonstrated in the inner limb (Niemi and Merenmies, 1960).

On the other hand, Hubbard (1954) has shown by measuring the oxygen consumption that the photoreceptor part of the rods does respire; the Q_{O_2} was found to be 1·0 and, hence, entirely comparable with that of many other tissues. Moreover, the respiration of the rod outer limb was found to be cyanide-sensitive, which led to the conclusion that it is mediated by the cytochromes.

Against this biochemical background, our present observation of abundant cytochrome oxidase activity in the outer segments of the photoreceptor cells is not unexpected. On the other hand, in our previous work (Niemi and Merenmies, 1960) DPN-diaphorase as well as alcohol dehydrogenase activities were found to be in the inner segments only, which makes the suggestion of Hubbard that the outer limb *in vivo* contains sufficient DPN to maintain the concentration of rhodopsin required

for vision, highly questionable. The role of the cytochrome respiration in the outer limb remains unexplained by the present findings.

One of the main drawbacks of the histochemical 'Nadi' reaction for cytochrome oxidase has been the great affinity of the reaction product for the lipids. The indophenol blue of the original method and indonaphthol purple of the modification of NACHLAS *et al.* (1958) are very lipid soluble, but the indoaniline and azamethine dyes produced in BURSTONE's (1959) modifications are said to be lipid insoluble. It does not, therefore, seem likely that the stain which we found in the rod and cone outer limbs is due to diffusion into the lipids, although these structures are known to be rich in lipid substances; our results with sections treated with cold acetone supply further evidence against this artefact. However, LILLIE (1952) has found two types of histochemically demonstrable lipid substances in the acromeres of the retinal rods; one of these was poorly soluble in organic solvents, being presumably a galactolipoprotein complex. Therefore, the possibility of the staining of lipids by our methods cannot be neglected.

We were unable to produce any staining of the distal segments by incubation of the retinal sections in solutions in which liver enzymes gradually liberated precipitate-forming groups; this, together with the instantaneous inhibition of the reaction by cyanide, can be taken as further evidence for the specificity of the results. Thus, we can conclude that the positive 'Nadi' reaction in the outer segments of the rods and cones is probably due to a true cytochrome oxidase activity. This result is in agreement with the findings of AKIYA (1952). He also found succinic dehydrogenase activity in the outer parts of rods and cones and put forward an idea that a mitochondria-like substance is present in the outer parts of the photosensitive cells.

The identification of cytochrome oxidase in the inner segment of rods and cones was not surprising because of the presence of high succinic dehydrogenase activity in this part of the sensory cells. In the other inner layer of the retina the cytochrome oxidase exhibited marked species differences, which corresponded generally to the differences in the distribution of succinic dehydrogenase. Thus, in the guinea pig and the rabbit, the cytochrome oxidase activity was, like succinic dehydrogenase, present in the photoreceptor cells only. However, the ganglion cell layer, which does not exhibit any activity of the latter enzyme, did show some cytochrome oxidase activity.

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SUMMARY

The distribution of cytochrome oxidase in the retina of the frog, the mouse, the rat, the guinea pig and the rabbit were studied using a modified histochemical 'Nadi' reaction. The reaction obtained was most intense in the outer segments of the photo-receptor cells. The significance of this observation is discussed in relation to experiments made to exclude non-enzymic staining. The inner segments of the rods and cones showed cytochrome oxidase activity, as did the outer plexiform layer in most species. The staining of the inner plexiform layer and of the ganglion cells was generally weak but varied considerably between different animal species.

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CHOLINESTERASE ACTIVITY AND PROTEIN CONTENT OF RAT BRAIN*

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AS PART of our programme of investigating the enzymic control of behaviour in the rat, we have already completed and reported studies on both cholinesterase (ChE) activity and lactic dehydrogenase (LDH) levels. Reliable individual, strain, and age differences in ChE activity of the rat were demonstrated (BENNETT *et al.*, 1958*a*), and a systematic relationship was found between differences in ChE activity and adaptive behaviour (ROSENZWEIG, KRECH and BENNETT, 1958). A comparison of LDH and ChE activities clearly indicated that the distribution of LDH and ChE activities in the cortex and subcortical brain of the rat differed in many significant respects (BENNETT *et al.*, 1958*b*). Of particular interest were the low correlation between ChE and LDH among the animals, the absence of strain differences in LDH levels, and the lack of any relationship between LDH levels and behaviour. These results were interpreted as supporting our hypothesis that in our previously observed correlation between ChE activity and behaviour, ChE activity is not simply an index to general enzymic or metabolic levels of the brain, but is rather a reflection of the fact that the acetylcholine–cholinesterase (ACh–ChE) system is of peculiar significance in the metabolism of the brain because of its contribution to synaptic transmission.

The present paper presents an additional test of this hypothesis. Inasmuch as ChE is a protein, the question can be raised whether the relationship between behaviour and ChE activity merely reflects the relationship of brain protein to behaviour rather than the specific role of ACh–ChE in synaptic transmission. To answer this question, protein and ChE analyses were made of the brains of rats from six different strains of animals with known ChE activity levels, and the ChE levels and the concentration of protein were compared within individual animals and among strains.

METHODS

The concentration of protein and ChE activity were determined for the visual (V) and somesthetic (S) areas of the cerebral cortex and of the subcortical brain (brain with dorsal cortex removed). To give a single value for cortical measures of percentage of protein and ChE activity, the determinations for the V and S areas were averaged $[(V + S)/2]$. Two hundred and eleven animals were tested for both percentage of protein and ChE activity and an additional 15 animals were tested for protein only. These 226 animals ranged in age from 6 days to 470 days at time of sacrifice, and were drawn from six strains of rats (two Tryon and four Roderick strains) developed and maintained in the Department of Psychology. The Tryon strains (the S_1 and the S_3 strains) were developed from a heterogeneous foundation stock by TRYON (1940) through selective breeding for maze brightness

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(the S_1 strain) and for maze dullness (the S_3 strain). The brain ChE activity of the S_1 strain has consistently been significantly higher than that of the S_3 strain. The four Roderick strains were developed by a selective breeding programme with the cortical ChE of the sire as the basis of selection (RODERICK, 1958). One set of these strains was derived from the Dempster, and one from the Castle foundation stock of the University of California Genetics Laboratory. Within each set, one strain has a considerably higher ChE activity than the other. These four strains are therefore labelled as the RDH (Roderick Dempster High ChE) and RDL, RCH, and RCL strains. Together with the Tryon rats we thus have three sets of animals, each deriving from a different foundation stock, and each set further subdivided by selective breeding into strains of high and low brain ChE activity.

Cholinesterase activity was determined by the rate of hydrolysis of acetylcholine in an automatic titrator, and is expressed in terms of moles $\times 10^{10}$ acetylcholine hydrolysed/min per mg tissue. The methods of preparation and analysis of tissue samples have been reported in detail (ROSENZWEIG *et al.*, 1958).

Protein content was determined with the Folin phenol reagent as described by LOWRY *et al.* (1951). Duplicate portions of the saline homogenates of the rat cortex or sub-cortical brain containing 1 mg wet tissue were mixed with 5 ml of the alkaline copper sulphate Reagent C in 12 ml tubes. After 10 min 0·50 ml of the diluted Folin–Ciocalteau phenol reagent was added and rapidly mixed. The optical density at 750 mμ was determined in 1 cm cells in a Beckman DU Spectrophotometer after 45 min. Suitable portions of a freshly diluted crystalline bovine albumin solution (Armour) were run with each series as standards. All results were referred to this standard (percentage of protein = percentage of nitrogen $\times$ 6·25), and the protein values are expressed as per cent of wet tissue weight. The absolute amount of protein may be slightly different from the values reported as the colour intensity for different proteins may not be the same as that obtained from the standard albumin. In addition, a small proportion (<5 per cent) of the colour is probably due to non-proteinaceous material (LOWRY *et al.*, 1951). However, within the rat cortex and brain, deviations from the correct absolute value are undoubtedly small, and relative differences found are significant.

RESULTS

The results show several significant differences between the distributions of ChE activity and percentage of protein in the rat brain (Tables 1–3*, Figs. 1 and 2):

(1) The relative values in the *V* and *S* areas of the cortex differ for the two measures.

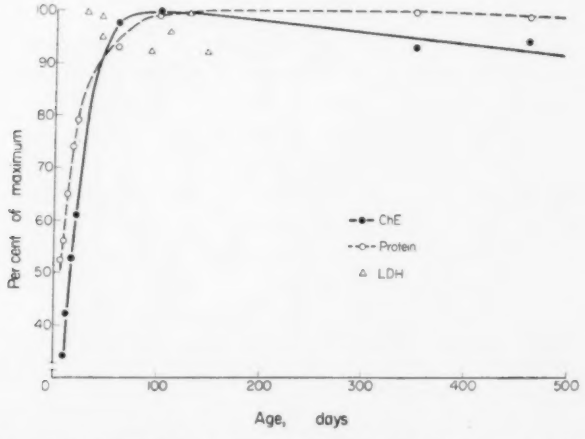

FIG. 1.—Cortical percentage of protein, cholinesterase activity, and lactic dehydrogenase activity as a function of age. Values are expressed as percentages of the maxima observed.

* Some of the groups in Tables 1, 2 and 3 representing a particular age and strain include subgroups that were either given different behavioural treatment or were analysed at different times. Since all subgroups of a particular age and strain showed closely similar values, results were pooled.

In previous studies we have consistently found ChE activity in the *S* area to be greater than that in the *V* area for all strains. In the present study this is true for 205 out of 211 animals; overall, ChE activity is 17 per cent greater in the *S* area than in the *V* area. For the percentage of protein, the reverse is true. This measure is higher in the *V* than in the *S* area for 165 out of the 226 animals tested. The mean percentage of protein for the *V* area is 10·11; the mean for the *S* area is 9·83. Thus, the protein value for the *S* area is 97 per cent of the protein value of the *V* area. While this difference is small, it is found in each of the six strains, and it is statistically significant in five of the six strains. The difference is significant at the 0·001 level for the S_1, S_3, RCL, and RCH strains, and at the 0·05 level for the RDH strain; it is not significant for the RDL strain.

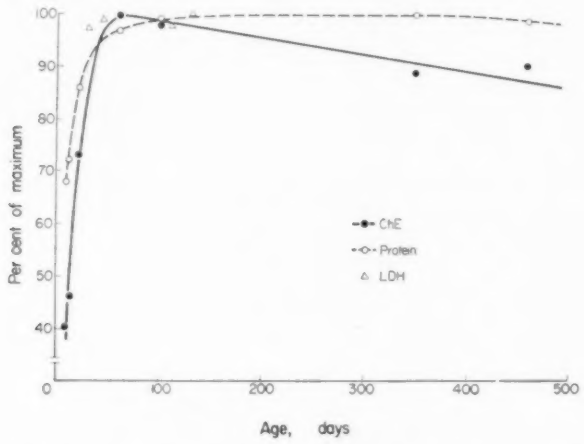

FIG. 2.—Subcortical percentage of protein, cholinesterase activity, and lactic dehydrogenase activity as a function of age. Values are expressed as percentages of the maxima observed.

(2) The relative values in the cortex and subcortex differ strikingly for the two measures. While the percentage of protein is almost equal for these two parts of the brain, cortical ChE activity is only about two-fifths as great as subcortical ChE activity. Values of the cortical–subcortical ratios for ChE and percentage of protein (Table 4A and B) indicate a high consistency among different strains and ages. For both ChE and percentage of protein, there is some increase in the cortical–subcortical ratio with age.

(3) Cholinesterase activity tends to vary more with age (within the limits studied) than does the percentage of protein (Figs. 1 and 2). At 10 days of age, cortical ChE activity is only about 35 per cent of the maximum value it will attain, while the concentration of protein is already at about 55 per cent of its maximum. Similarly, subcortical ChE activity at 10 days is at about 40 per cent of its maximum, while the concentration of protein is already at about 70 per cent of its maximum value. Once the percentage of protein reaches its maximum at about 100 days of age, it tends to show very little further change. Cholinesterase activity, on the contrary, tends to decline from its peak values, which occur for the cortex at about 100 days and for the subcortex at about 60 days of age. The change of protein content with age in the rat brain is similar to that reported by FOLCH-PI (1955) for mouse brain.

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TABLE 1.—COMPARISONS BETWEEN CHOLINESTERASE ACTIVITY AND THE PERCENTAGE OF PROTEIN IN BRAIN OF S_1-RATS AT DIFFERENT AGES

Age (days)	N		Cholinesterase activity*				Percentage of protein†			
			V	S	$\frac{V+S}{2}$	Subcortex	V	S	$\frac{V+S}{2}$	Subcortex
6	4	$\bar{X}$	—	—	—	—	5·86	5·52	5·68	—
		S.D.	—	—	—	—	0·20	0·31	0·12	—
10	10	$\bar{X}$	19·0	25·5	22·3	67·9‡	6·22	6·14	6·19	7·21§
		S.D.	2·7	2·4	1·9	12·0‡	0·44	0·36	0·30	0·07§
13	15	$\bar{X}$	23·8	31·2	27·4	77·5	7·11	7·12	7·12	7·65
		S.D.	1·8	2·2	1·7	4·0	0·45	0·59	0·44	0·13
17	6	$\bar{X}$	31·9	38·0	35·0	—	8·15	8·17	8·16	—
		S.D.	1·8	2·0	1·4	—	0·16	0·23	0·16	—
21	14	$\bar{X}$	34·7	45·3	40·1	125·1	8·77	8·50	8·64	9·06
		S.D.	2·0	3·6	2·6	5·2	0·25	0·77	0·42	0·10
~ 60	14	$\bar{X}$	59·8	68·9	64·3	170·3	10·41	10·13	10·27	10·28
		S.D.	2·9	3·2	2·5	7·9	0·13	0·38	0·20	0·11
~100	9	$\bar{X}$	61·5	69·7	65·6	166·8	10·95	10·98	10·97	10·46
		S.D.	2·7	2·9	2·4	4·6	0·16	0·17	0·14	0·13
~350	10	$\bar{X}$	57·6	64·6	61·1	151·1	11·06	10·91	10·99	10·59
		S.D.	2·2	2·1	1·6	4·2	0·16	0·33	0·23	0·15
~460	11	$\bar{X}$	58·7	64·9	61·8	153·0**	10·92	10·77	10·85	10·49
		S.D.	3·8	2·6	2·8	7·6**	0·29	0·34	0·27	0·16

* Expressed in terms of moles $\times 10^{10}$ acetylcholine hydrolysed/min per mg wet tissue.
† Expressed as per cent of wet tissue weight; bovine albumin used as the reference standard.
‡ $N = 2$.
§ $N = 3$.
** $N = 4$.

Figs 1 and 2 also include values for animals whose lactic dehydrogenase (LDH) and ChE activities were reported in a previous paper (BENNETT *et al.*, 1958*a*). The LDH values show very little change with age. In the cortex and subcortex, LDH had already achieved its maximum at the earliest age at which it was measured (30 days). The early achievement of its maximal level by LDH is consistent with its important role in anaerobic metabolism, which is of great significance for the embryo and neonate.

(4) We have previously noted the low biochemical variability of the brain as compared with other tissues (BENNETT *et al.*, 1958*b*). The variabilities of different measures can be compared by computing coefficients of variation for each measure [Coeff. Var. = 100 (S.D./$\bar{X}$)]. Coefficients of variation were compared for ChE activity and percentage of protein for each subgroup of rats in Tables 1, 2, and 3. For the cortex [$(V + S)/2$] this meant 19 comparisons; in every one, the variability

TABLE 2.—COMPARISONS BETWEEN CHOLINESTERASE ACTIVITY AND PERCENTAGE OF PROTEIN IN BRAIN OF S_3-RATS AT DIFFERENT AGES

Approx. age (days)	N		Cholinesterase activity*				Percentage of protein†			
			V	S	$\frac{V+S}{2}$	Subcortex	V	S	$\frac{V+S}{2}$	Subcortex
100	29	$\bar{X}$	52·1	65·7	58·9	156·6	10·87	10·58	10·72	10·43
		S.D.	2·7	4·2	3·1	5·3	0·16	0·24	0·15	0·13
370	8	$\bar{X}$	50·1	59·8	55·0	143·4	11·23	10·97	11·10	10·49
		S.D.	2·0	2·6	2·0	4·1	0·15	0·23	0·14	0·15
465	4	$\bar{X}$	52·5	60·2	56·3	142·5	10·89‡	10·78‡	10·83‡	10·55‡
		S.D.	2·2	1·1	1·6	10·1	0·23	0·24	0·19	0·10

* Expressed in terms of moles $\times 10^{10}$ acetylcholine hydrolysed/min per wg. wet tissue.
† Expressed as per cent of wet tissue weight; bovine albumin used as the reference standard.
‡ $N = 9$.

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of ChE activity was greater than that of percentage of protein. For ChE activity, the standard deviations (S.D.s) tended to be about 5 per cent of the means; for percentage of protein, the S.D.s tended to be only about 2 per cent of the means. For the subcortex, comparisons could be made for 18 groups; again the variability of ChE activity was greater than that of percentage of protein in every comparison.

TABLE 3.—COMPARISONS BETWEEN CHOLINESTERASE ACTIVITY AND PERCENTAGE OF PROTEIN IN BRAINS OF RATS OF RODERICK STRAINS

Strain	Approx. age (days)	N		Cholinesterase activity*				Percentage of protein†			
				V	S	$\frac{V+S}{2}$	Sub-cortex	V	S	$\frac{V+S}{2}$	Sub-cortex
RCL	250	9	$\bar{X}$	44·4	49·9	47·1	138·9	10·77	9·94	10·37	10·62
			S.D.	4·1	4·0	3·9	4·8	0·25	0·40	0·25	0·11
RCL	330	6	$\bar{X}$	40·6	47·4	44·0	119·8	10·65	10·15	10·40	10·51
			S.D.	2·2	2·2	1·4	4·0	0·06	0·30	0·17	0·13
RCH	250	6	$\bar{X}$	63·0	72·1	67·6	169·3	10·59	9·84	10·22	10·46
			S.D.	3·2	2·2	2·6	6·8	0·41	0·28	0·22	0·08
RCH	330	5	$\bar{X}$	60·1	65·6	62·9	150·2	10·74	10·05	10·39	10·72
			S.D	3·4	3·1	3·1	4·8	0·16	0·26	0·19	0·27
RDL	100	16	$\bar{X}$	48·0	51·2	49·7	132·0	10·82	10·65	10·74	10·70
			S.D.	3·6	2·9	2·9	6·0	0·37	0·40	0·26	0·14
RDH	100	19	$\bar{X}$	63·0	69·6	66·4	151·8	10·95	10·61	10·78	10·59
			S.D.	4·4	5·5	4·4	6·6	0·32	0·41	0·22	0·14

* Expressed in terms of moles $\times 10^{10}$ acetylcholine hydrolysed/min per mg wet tissue.
† Expressed as per cent of wet tissue weight; bovine albumin used as the reference standard.

TABLE 4.—CORTICAL–SUBCORTICAL RATIOS FOR CHOLINESTERASE AND PERCENTAGE OF PROTEIN A.—TRYON STRAINS, B.—RODERICK STRAINS

A

Age (days)	ChE		Protein %	
	S_1	S_2	S_1	S_2
10	0·33		0·86	
13	0·34		0·92	
21	0·32		0·95	
~ 60	0·38		1·00	
~100	0·38	0·37	1·05	1·03
~350	0·40	0·38	1·04	1·06
~460	0·40	0·40	1·03	1·03

B

Age (days)	ChE				Protein %			
	RCL	RCH	RDL	RDH	RCL	RCH	RDL	RDH
~100			0·38	0·44			1·00	1·02
~250	0·34	0·40			0·98	0·98		
~350	0·37	0·42			0·99	0·97		

The coefficients of variation averaged about 5 per cent for ChE activity, and only about 1 per cent for protein. Thus, from individual to individual within every subgroup, percentage of protein showed consistently less variability than did ChE activity. This was true for both the cortex and subcortex.

Correlations between the two cortical areas and between cortex and subcortex are moderately high and significant for ChE activity, and low and insignificant for percentage of protein. This is demonstrated by the first four columns of correlations in Table 5. For each subgroup that consisted of 8 or more rats, several correlations were computed (Table 5). First, the values obtained for the *V* area of the cortex were correlated with those for the *S* area. For 10 of the 12 subgroups, the correlation for ChE activity was greater than that for percentage of protein. The correlation coefficients in each column were then combined by using FISHER's *r* to *Z* transformation.

TABLE 5.—CHOLINESTERASE AND PROTEIN CORRELATIONS FOR VARIOUS PARTS OF RAT BRAIN

Strain	*N*	*V* vs. *S*		Subcortical vs. $\frac{V+S}{2}$		ChE vs. Protein %	
		ChE	Protein %	ChE	Protein %	$\frac{V+S}{2}$	Subcortical
S_1	10	0·20	0·73	0·10	−0·15	−0·14	−0·02
	8	0·82	0·08	0·18	0·40	−0·65	0·63
S_2	10	0·59	0·06	0·75	−0·15	0·57	−0·12
	10	0·79	0·05	0·66	−0·58	0·13	0·12
	8	0·44	0·11	0·16	0·66	0·71	0·40
RDH	9	0·77	−0·31	0·86	0·01	0·17	−0·49
	10	0·52	−0·25	0·71	−0·02	0·38	0·67
RDL	9	0·44	0·09	0·37	0·30	0·55	0·61
RCL	9	0·83	0·27	0·79	0·22	0·07	0·84
S_1 (Female)	9	0·60	0·62	−0·02	0·22	−0·08	0·45
	9	0·60	0·24	0·59	0·24	−0·46	0·14
	8	0·78	0·37	0·63	0·09	−0·36	0·38
Combined‡	109	0·64†	0·20	0·55†	0·08	0·10	0·33*

* Significant at 0·05 level.
† Significant at 0·01 level.
‡ Correlations for combined groups are weighted averages using FISHER's *r* to *Z* transformation.

For ChE activity, the combined correlation coefficient is 0·64. For percentage of protein, the combined coefficient does not differ significantly from zero. Similar findings hold for relationships between cortical and subcortical values. Again, the ChE activity tends to be correlated between cortex and subcortex, while the percentage

TABLE 6.—DIFFERENCES BETWEEN PAIRED STRAINS IN CHOLINESTERASE ACTIVITY AND PERCENTAGE OF PROTEIN

Strain	Approx. age (days)	Cholinesterase activity				Percentage of protein			
		N	*V*	*S*	Sub-cortical	*N*	*V*	*S*	Sub-cortical
S_1	100	9	61·51	69·66	166·8	9	10·95	10·98	10·46
S_3	100	29	52·11	65·68	156·6	29	10·87	10·58	10·43
Diff.			9·40‡	3·98*	10·2‡		0·08	0·40‡	0·03
S_1	350	10	57·61	64·57	151·1	10	11·07	10·91	10·59
S_3	350	8	50·08	59·85	143·4	8	11·23	10·97	10·49
Diff.			7·53‡	4·72‡	7·7*		−0·17*	−0·06	0·10*
S_1	460	5	58·68	64·92	153·0§	11	10·92	10·77	10·49
S_3	460	4	52·48	60·25	142·5	9	10·89	10·78	10·55
Diff.			6·20*	4·67*	10·5		0·03	−0·01	−0·06
RCH	250	6	63·02	72·13	169·33	6	10·59	9·84	10·46
RCL	250	9	44·36	49·87	138·89	9	10·77	9·94	10·62
Diff.			18·66‡	22·26‡	30·44‡		−0·18	−0·10	−0·16*
RCH	330	5	60·08	65·64	150·20	5	10·74	10·05	10·72
RCL	330	6	40·62	47·37	119·83	6	10·65	10·15	10·51
Diff.			19·46‡	18·27‡	38·37‡		0·09	−0·10	0·21
RDH	100	19	63·02	69·60	151·8	19	10·95	10·61	10·59
RDL	100	16	48·02	51·23	132·0	16	10·82	10·65	10·70
Diff.			15·00‡	18·37‡	19·8‡		0·13	−0·04	−0·11*

* Significant at 0·05 level of confidence.
† Significant at 0·01 level of confidence.
‡ Significant at 0·001 level of confidence.
§ $N = 4$.

of protein lacks such correlation. As has been reported previously (BENNETT *et al.*, 1958*b*), the ChE correlations between the two cortical areas tend to be greater than those between cortex and subcortex. In the present case, both correlations, combined for the total group, were significant.

In order to determine whether ChE activity bears any relation to the percentage of protein, correlations between these two measures were computed. Column 5 of Table 5 indicates that the two measures show no consistent relationship in the cortex. Column 6 indicates that there is a moderately low and statistically significant relationship between ChE activity and percentage of protein in the subcortex. Thus, from individual to individual within a subgroup, cortical ChE activity varies independently of cortical protein concentration. Subcortical ChE activity seems to be related to subcortical protein concentration, but the relationship is moderately low.

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Within strains the range of variability of both ChE and percentage of protein is restricted, making it somewhat difficult to find relationships between them; this may explain the lack of correlations observed. Since this difficulty arises in analysis *within* strains, comparisons were made *between* strains. It will be recalled that three pairs of strains were used, each consisting of one high-ChE and one low-ChE strain. The differences in ChE activity *between* paired strains are relatively large, and we therefore looked for possible differences *between* these paired strains in percentage of protein. Table 6 presents means of ChE activity and percentage of protein for paired high- and low-ChE groups matched in age. The differences between means of comparable groups were obtained, and the significance of each difference was computed by means of the *t*-test (Table 6). The left half of the Table shows that the paired groups differ markedly in ChE activity. All of the 18 differences are in the expected direction; 13 of them are significant at the 0·001 level of confidence, one is significant at the 0·01 level, three are significant at the 0·05 level, and only one difference is not significant (and this one is based on a very small number of samples). The right half of the table shows a much less consistent and clear-cut picture. In 8 instances, the subgroup with higher ChE activity also has the higher percentage of protein, but in 10 instances the difference is in the opposite direction.

DISCUSSION

The results of the present study, in which the percentage of protein is compared with ChE activity in rat brain, are similar in all major respects to those of our previous study which compared LDH levels with ChE activity. First, for both sets of comparisons it appears that the distribution of ChE activity in the cortex and subcortical brain of the rat differs in many significant ways from either percentage of protein or LDH. Secondly, in the present study were found generally low and inconsistent correlations between ChE and the percentage of protein; no significant differences in the percentage of protein between strains of animals which differed significantly in ChE activity; and lower individual variability in percentage of protein than in ChE. All of these findings are strikingly analogous to our comparisons of ChE and LDH.

Both sets of findings, taken together, can be taken to support the interpretation that the previously observed correlation between ChE activity and behaviour reflects the *specific* significance of the ACh–ChE system in brain metabolism because of its contribution to synaptic transmission. Cholinesterase activity cannot be taken merely as an index to either general enzymic activity in the brain or general protein level.

In view of our general hypothesis of the relationship between ChE and behaviour, it should be noted that of all the chemical or morphological measures of rat brain which we have thus far analysed, ChE activity shows the largest degree of individual variability. If ChE is significantly implicated in accounting for individual differences in behaviour, it is encouraging that ChE itself shows relatively large individual differences.

SUMMARY

Protein concentration and cholinesterase (ChE) activity were determined for six strains of rats. These measures differ in the following respects. (1) Relative values for visual and somesthetic cortex, and subcortex, differ for the two measures. (2) Cholinesterase activity is the more variable with age. (3) Individual variability is greater for ChE. (4) Cholinesterase values are significantly correlated from locus to

locus within brain, while protein values are not. (5) Percentage of protein and ChE show no correlation with each other in cortex, and only a moderately low correlation in subcortex. (6) Pairs of strains differing markedly in ChE activity do not differ in percentage of protein.

These results are interpreted to mean that in correlations observed between ChE activity and behaviour, ChE is not simply an index to the general brain metabolism.

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THE EFFECTS OF PYRIDOXINE ANTAGONISTS AND OTHER CONVULSIVE AGENTS ON AMINO ACID CONCENTRATIONS OF THE MOUSE BRAIN*

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A SEIZURE state has been produced by pyridoxine deprivation in lower mammals and man (HUNT *et al.*, 1954; COURSIN, 1954). These seizures are believed to result from an inhibition of the enzymic processes of the brain in which pyridoxine functions as a co-enzyme (ROBERTS *et al.*, 1958*a*) and which act mainly to metabolize glutamic acid through the decarboxylation of glutamic acid to γ-aminobutyric acid (GABA) and the transamination of glutamic acid with other α-amino acids. The relationship of the metabolic defects induced by this enzymic inhibition to the evoked seizures is, however, not known.

GAMMON *et al.* (1960) used 4-methoxymethylpyridoxine (MOB6), a specific pyridoxine antagonist, to produce seizures in mice through acute pyridoxine deficiency. They found that seizures caused by MOB6 could be prevented by prior treatment of the animals with pyridoxine or GABA. The present investigation was undertaken to elucidate the amino acid changes which occur during both the seizure state produced by pyridoxine deficiency and the prevention of this state by protecting agents. In addition, several other convulsive agents were studied to determine if their effect on amino acid metabolism might parallel that seen during the seizure state induced by pyridoxine deficiency.

MATERIALS AND METHODS

Male Swiss white mice weighing 20 g were tested in nine experimental situations as follows.

Control series. At least 6 normal control animals were sacrificed during each experimental period.

Pyridoxine series. Pyridoxine hydrochloride in a dose of 1·8 mg per mouse was administered to 18 animals, and the animals were sacrificed 1 hr later.

γ-Aminobutyric acid series. GABA in a dose of 100 mg per mouse was administered to 18 animals, and the animals were sacrificed 1 hr later.

4-Methoxymethylpyridoxine series. MOB6 was administered in a dose of 0·6 mg per mouse, the convulsant dose 100 (CD_{100}), in 16 animals and 1·2 mg per mouse, twice the CD_{100}, in 5 animals. The animals were sacrificed during a seizure.

Pyridoxine–MOB6 series. Pyridoxine hydrochloride was administered in a dose of 1·8 mg per mouse in 18 animals followed in 15 min by administration of 0·6 mg of MOB6 per mouse. The animals were sacrificed 45 min after MOB6 administration.

GABA–MOB6 series. GABA in a dose of 100 mg per mouse was administered to 18 mice followed in 15 min by administration of 0·8 mg of MOB6 per mouse. Animals were sacrificed while in seizure. Each of a second group of 12 mice was injected with 100 mg of GABA followed in 15 min by 0·6 mg of MOB6. These animals did not convulse and were sacrificed 45 min after the MOB6 administration.

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Pentamethylenetetrazol (Metrazol) series. Pentamethylenetetrazol in a dose of 6 mg per mouse, twice the lethal dose 100 (LD_{100}) (CHEN and BOHNER, 1958), was administered to 19 animals which were sacrificed while in seizure.

Picrotoxin series. Picrotoxin in a dose of 0·3 mg per mouse, twice the LD_{50} (CHEN and BOHNER, 1958), was administered to 17 animals which were sacrificed while in seizure.

Tripellenamine (Pyribenzamine) series. Tripellenamine in a dose of 2·8 mg per mouse, twice the LD_{50} (LEHMANN, 1948), was administered to 17 animals which were sacrificed while in seizure.

Electrical stimulation series. Square wave electrical stimulation with a tetanizing current was utilized to induce seizures in 24 animals. Electrodes were attached to one ear and the tail of each mouse. Movements of the mice were not restrained while the animals were in seizure. The animals were sacrificed after 30 sec of continuous seizure.

All the experimental drugs were administered by intraperitoneal injection. No anaesthetics were used during any part of the experiment.

The animals were sacrificed by dislocation of the cervical vertebrae. The calvarium was opened with scissors, and the entire brain including the brain stem removed and placed in a vial kept cold in a dry-ice–95% ethanol bath. Removal and freezing of the brains took 15–20 sec. From 4 to 7 brains were pooled and, after weighing of the sample, homogenized in 12 ml of cold absolute ethanol. The homogenizer was washed with 15 ml of absolute ethanol and 5 ml of distilled water. The sample and wash were centrifuged and treated with 10 ml of absolute ethanol. The supernatant solution was treated with twice its volume of chloroform and centrifuged. The resulting aqueous layer was removed, evaporated to near dryness, and redissolved in 1 ml of water. The final sample was clear and yellow.

Five μg of the sample, representing approximately 12 mg of wet weight brain tissue, were spotted on an 8 × 8 inch square of Whatman #1 filter paper. Two-dimensional ascending paper chromatography was performed with *n*-butanol–glacial acetic acid–distilled water, 100:25:50, as the first solvent phase and redistilled phenol–distilled water, 100:40, as the second solvent phase. Colour development was performed with 0·3% ninhydrin in 95% ethanol (STEPKA, 1957). The amino acid spots were cut out, treated with 0·01% cadmium chloride in 60% methanol (BLOCK *et al.*, 1958), and the optical density of the resultant solution was determined with a Klett–Summerson photoelectric colorimeter with a green filter. All chromatographs were run in duplicate. The optical density readings in which the results are expressed were extrapolated to values expected from 12 mg of wet brain tissue. Since variations in colour development from day to day are inherent in this system, direct comparison of the optical density readings among the different series cannot be made (MOORE and STEIN, 1948). For this reason, control samples of normal mouse brain were included in each chromatographic run. Statistical analysis of the data was performed. Results significant to between the 5% and 1% levels and those significant to less than the 1% level are indicated.

RESULTS

The amino acids separated by the chromatographic technique used here were GABA, glutamine, glutamic acid, aspartic acid, alanine, glutathione, and a mixture of glycine, serine, and taurine which could not be separated further by our procedure. Alanine and glutathione were present in concentrations too small to be analysed quantitatively and will not be reported.

Control series. Control animals were run concomitantly with each experimental series. The optical density readings representing the normal values for the amino acids of the control brains are listed with the experimental results in each table. Although the proportions among the various amino acids remain constant, the absolute optical density readings differ greatly because of the day to day variation in such factors as temperature and humidity (MOORE and STEIN, 1948). The relative proportions of GABA to glutamine and glutamic acid are similar to those reported in analyses of rat brain (ANSELL and RICHTER, 1954). Glutamic acid is always the preponderant amino acid in the untreated mouse brain, and glutamine is usually present in the second highest concentration (Tables 1, 2 and 3).

Pyridoxine series. Pyridoxine caused no overt changes in the behaviour of the

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animals during the experimental period. Analyses, however, revealed changes in three amino acids (Table 1). The concentration of GABA increased while glutamine and aspartic acid concentrations decreased.

GABA series. The behaviour of the animals following massive GABA administration was not overtly changed from normal. Amino acid analysis showed a marked, two-fold increase in the GABA content of the brain as well as a lesser increase of aspartic acid (Table 1).

TABLE 1.—THE EFFECT OF EXPERIMENTAL PROCEDURES ON AMINO ACIDS IN THE MOUSE BRAIN*

	Pyridoxine			GABA			4-Methoxymethylpyridoxine					
							LD_{100}			Twice LD_{100}		
	Cont.	Exp.	*P*	Cont.	Exp.	*P*	Cont.	Exp.	*P*	Cont.	Exp.	*P*
GABA	28 ± 0	34 ± 2	<0·05	15 ± 1	34 ± 2	<0·01	43 ± 3	22 ± 1	<0·01	43 ± 3	23 ± 0	<0·01
Glutamine	120 ± 3	103 ± 4	<0·01	61 ± 4	61 ± 5	>0·05	105 ± 3	104 ± 3	>0·05	105 ± 3	108 ± 1	>0·05
Glutamic acid	207 ± 10	186 ± 5	>0·05	143 ± 8	156 ± 7	>0·05	199 ± 14	195 ± 3	>0·05	199 ± 14	210 ± 4	>0·05
Glycine, Serine, Taurine	102 ± 3	116 ± 7	>0·05	63 ± 13	58 ± 3	>0·05	81 ± 4	80 ± 3	>0·05	81 ± 4	86 ± 1	>0·05
Aspartic acid	81 ± 5	59 ± 5	<0·05	38 ± 1	48 ± 2	<0·01	22 ± 3	16 ± 1	>0·05	22 ± 3	25 ± 1	>0·05
No. of animals	6	18		6	18		18	16		18	5	

* All readings are expressed in mean optical density readings and are ± the mean deviation.
Cont. = control; Exp. = experimental; *P* = probability.
LD_{100} = lethal dose 100; Twice LD_{100} = two times the lethal dose 100.

MOB6 series. Clonic tonic convulsions occurred in all animals within 20 to 40 min after MOB6 administration. The animals were allowed to convulse intermittently for 10 min before being sacrificed. Similar results of essentially equal magnitude were observed following administration of MOB6 in the two different doses. In both series, the only amino acid change observed was a decrease in GABA concentration to one half of its expected value (Table 1).

Pyridoxine–MOB6 series. Pyridoxine administered in a dose three times that of MOB6 will effectively block the seizures caused by MOB6 (GAMMON *et al.*, 1960). Thus, no seizures were observed following the sequential administration of these drugs nor was the behaviour of the animals changed in any way. Despite the absence of seizures, a decrease in GABA values to one-half their normal level was observed (Table 2). This change parallels that seen after administration of MOB6 alone. The amino acid changes caused by administration of pyridoxine alone were not observed.

GABA–MOB6 series. GABA has previously been reported to prevent MOB6 induced seizures (GAMMON *et al.*, 1960). In this series GABA given in a dose of 100 mg per mouse prevented seizures from occurring when MOB6 was administered at the CD_{100} level. Administration of GABA at 100 mg per mouse followed by MOB6 at a dose greater than the CD_{100} level resulted in tonic-clonic seizures which occurred 25–40 min following the MOB6 administration. GABA levels much above 100 mg per mouse are lethal (SAMSON *et al.*, 1959). Therefore, higher concentrations of GABA could not be used to block these seizures. When MOB6 and GABA were given

concomitantly, the animals appeared lethargic whether they went into seizure or were protected.

The presence or absence of seizures evoked different amino acid patterns in the mouse brain (Table 2). In both cases glutamine and GABA concentrations were

TABLE 2.—THE EFFECT OF EXPERIMENTAL PROCEDURES ON AMINO ACIDS IN THE MOUSE BRAIN

	Pyridoxine–MOB6			GABA–MOB6					
				Seizure			No seizure		
	Cont.	Exp.	*P*	Cont.	Exp.	*P*	Cont.	Exp.	*P*
GABA	37 ± 3	20 ± 2	**<0·01**	28 ± 1	39 ± 3	**<0·01**	29 ± 2	89 ± 5	**<0·01**
Glutamine	82 ± 9	96 ± 5	>0·05	69 ± 4	91 ± 6	**<0·05**	61 ± 4	75 ± 4	**<0·05**
Glutamic acid	188 ± 5	176 ± 9	>0·05	145 ± 24	153 ± 12	>0·05	111 ± 12	162 ± 12	**<0·05**
Glycine, Serine, Taurine	91 ± 3	77 ± 6	>0·05	63 ± 1	49 ± 5	**<0·05**	34 ± 2	49 ± 3	**<0·01**
Aspartic acid	37 ± 1	36 ± 2	>0·05	14 ± 2	15 ± 2	>0·05	7 ± 0	11 ± 2	>0·05
No. of animals	6	18		6	18		12	12	

increased above normal. In addition, animals having no seizures exhibited increases in the concentrations of glutamic acid and glycine, serine and taurine; convulsive animals showed a decrease in the glycine, serine, and taurine group.

Pentamethylenetetrazol series. Convulsions occurred in all animals in this series 30–40 sec following administration of pentamethylenetetrazol. Clonic movements were observed for 5–10 sec followed by a tonic extensor seizure which evolved in 1–3 sec into a tonic flexor seizure. Animals were sacrificed in this last stage. No change in the amino acid concentrations was observed (Table 3).

Picrotoxin series. Clonic extensor seizures were observed approximately 5 min following administration of picrotoxin. Intermittant seizures were allowed to continue for 15 min before the animals were sacrificed. No changes in amino acid concentrations in the brain were observed (Table 3).

Tripellenamine series. Clonic-tonic convulsions started approximately 1 min following administration of tripellenamine. The animals were allowed to remain in *status epilepticus* for 30 sec and were sacrificed while still in seizure. Analysis of amino acids demonstrated decreases in GABA, glutamine, and aspartic acid concentrations (Table 3).

Electrical stimulation series. Animals were kept in continuous tonic extensor seizure for 30 sec and sacrificed while still in seizure. Chromatographic analysis demonstrated an increase in glutamine concentration in the brain (Table 3).

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TABLE 3.—THE EFFECT OF EXPERIMENTAL PROCEDURES ON AMINO ACIDS IN THE MOUSE BRAIN*

	Pentamethylenetetrazol			Picrotoxin			Tripellenamine			Electroshock		
	Cont.	Exp.	*P*	Cont.	Exp.	*P*	Cont.	Exp.	*P*	Cont.	Exp.	*P*
GABA	42 ± 3	37 ± 5	>0·05	29 ± 1	31 ± 1	>0·05	37 ± 1	33 ± 1	<0·05	35 ± 1	36 ± 1	>0·05
Glutamine	101 ± 4	85 ± 8	>0·05	79 ± 0	79 ± 4	>0·05	101 ± 8	79 ± 1	<0·05	73 ± 2	88 ± 2	<0·01
Glutamic acid	184 ± 14	117 ± 23	>0·05	155 ± 17	154 ± 5	>0·05	167 ± 7	169 ± 8	>0·05	150 ± 10	165 ± 5	>0·05
Glycine, Serine, Taurine	75 ± 4	67 ± 7	>0·05	44 ± 1	43 ± 1	>0·05	62 ± 1	55 ± 3	>0·05	47 ± 6	62 ± 6	>0·05
Aspartic acid	21 ± 2	23 ± 6	>0·05	2 ± 0	3 ± 1	>0·05	34 ± 2	26 ± 1	<0·05	13 ± 3	23 ± 4	>0·05
No. of animals	24	19		6	17		6	17		12	24	

DISCUSSION

Analysis of selected amino acids of the mouse brain following the administration of various convulsive and anti-convulsive drugs showed no consistent changes in the amino acid profiles by which one could predict the excitatory level of the brain. Even in those series where the action of MOB6 was specifically inhibited by administration of pyridoxine, the amino acid changes did not correlate with the observed change in the excitatory state of the animals as seen by the presence or absence of seizures (Table 2). The results of the different experimental series must thus be discussed separately as no unifying principle is seen to underly them.

The role of glutamic acid and its metabolites, particularly GABA, in the regulation of the excitatory state of the brain has been suggested in recent years. The experiments reported here, as well as those reviewed by ROBERTS *et al.* (1958*b*) and ELLIOTT and JASPER (1959) demonstrate that the seizure state is accompanied by no consistent change in the amino acids of the brain when a variety of convulsive agents are used to produce seizures. These studies also indicate inconsistent results when glutamic acid or its metabolites are used to inhibit seizures caused by various convulsive agents. This present experiment demonstrates that both pyridoxine and GABA can inhibit and prevent MOB6-induced seizures (Table 2).

MOB6 is believed to act as a specific pyridoxine antagonist and to have no other pharmacologic effects. Since pyridoxine acts as a co-enzyme for L-glutamic acid decarboxylase and glutamic acid transaminase, inhibition of pyridoxine will inhibit the action of these enzymes. Of these enzymes, L-glutamic acid decarboxylase is the most strongly inhibited by pyridoxine antagonists because pyridoxine is loosely bound to the apoenzyme and thus more easily displaced (ROBERTS *et al.*, 1958*a*). It is suggested that the decrease in GABA concentration of the brain seen after MOB6 administration is a direct result of inhibition of L-glutamic acid decarboxylase since the elaboration of GABA is achieved in large part through the action of this enzyme.

It has been reported previously that pyridoxine will protect mice from MOB6-induced seizures (GAMMON *et al.*, 1960). The amino acid changes induced by pyridoxine (Table 1) are probably accounted for by the observation that in the normal brain the amount of pyridoxal phosphate available is less than that needed for maximal functioning of the decarboxylases of the brain (DE MARCO, 1957). Saturation of the decarboxylases with exogenous pyridoxine, then, increases their activity and can result in the amino acid changes observed.

When MOB6-induced seizures were prevented by concomitant administration of pyridoxine, the effects produced paralleled that produced by MOB6 alone and did not resemble the effects observed by pyridoxine alone (Table 2). Thus, an identical amino acid pattern is seen to be present whether MOB6 seizures are present or are prevented by pretreatment with pyridoxine. It must then be concluded that the concentration of GABA in the whole brain cannot be used as an index of the state of brain excitability. That the concentration of GABA in specific areas of the brain might reflect brain excitability by appropriate local changes remains as an interesting speculation.

Since glutamic acid and its metabolites protect against seizures induced by various convulsive agents, the question arises of whether this protection is a non-specific pharmacologic depression of central nervous system excitability or a specific repair of a deficit of these metabolites. It has been demonstrated that convulsive doses of MOB6 significantly lower the GABA concentration in the mouse brain. When GABA was administered concomitantly with MOB6 and seizures thereby prevented the GABA content of the brain was increased by 300 per cent (Table 2). When seizures could not be prevented because of the use of larger doses of MOB6 with GABA, an elevation of the concentration of GABA in the brain was still observed though of the much smaller magnitude of 30 per cent (Table 2). The 30 per cent increase in GABA above normal control concentration, however, represents a decrease from the expected concentration since exogenous GABA administered alone will cause a 200 per cent increase in GABA concentration in the brain (Table 1). Although it has been reported that GABA does not pass the blood–brain barrier (ELLIOTT and JASPER, 1959), the concentration of GABA administered in this experiment is much in excess of that utilized by previous workers. It may, therefore, be suggested that this massive dose of GABA is sufficient to overcome whatever resistance the brain might have to the entrance of this compound. Thus, under conditions where GABA does protect against MOB6-induced seizures, the concentration of GABA in the brain is greater than that seen when GABA alone is administered. Conversely, when GABA does not protect against MOB6-induced seizures, its concentration in the brain is less than that produced by GABA alone although it is still above normal control levels. This series again demonstrated that the absolute elevation or depression from the normal control values of GABA concentration in the brain cannot be equated with the presence or absence of seizures. These results further confirm that the changes of GABA concentration in the whole brain cannot be used to predict or characterize the excitatory state of the brain.

Seizures produced by pentamethylenetetrazol or picrotoxin administration resulted in no changes in amino acid levels in the brain; this result confirms the work of KILLAM and BAIN (1957) and RICHTER and DAWSON (1948) respectively (Table 3). Analysis of the brains of animals having seizures induced by tripellenamine, however, demonstrated significant amino acid decreases (Table 3). Although the possible significance of this result cannot be explained at this time, it is of interest to note that tripellenamine is utilized to potentiate seizure activity in human epileptics (CHURCHILL and GAMMON, 1949). Electroshock-induced convulsions also resulted in an amino acid change (Table 3). This is contrary to previous reports of DAWSON (1953) and RICHTER and DAWSON (1948) who observed no change in amino acid concentrations in the rat brain following electroshock.

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The question of the role of glutamic acid and its metabolites in the protection from seizures cannot be answered as yet. The importance of local changes of GABA in small areas of the brain as opposed to general changes in the entire brain, as was discussed by ELLIOTT and JASPER (1959), cannot be evaluated by the experimental scheme utilized here. It would appear that at present one can offer no consistent correlation between the excitatory state of the brain and the changes in selected amino acids of the brain.

SUMMARY

1. The effects of the intraperitoneal administration to mice of several convulsive and anticonvulsive agents have been studied by amino acid analysis of the brain.

2. MOB6 decreased GABA to one-half its normal concentration following seizures. Protection from MOB6-induced seizures with pyridoxine did not affect this GABA decrease. Protection from MOB6-induced seizures with GABA resulted in a large increase in GABA concentration. Seizures caused by pentamethylenetetrazol and picrotoxin caused no amino acid changes. Tripellenamine-induced seizures decreased GABA and glutamine concentrations, and electroshock seizures increased glutamine concentration.

3. No common amino acid patterns emerged which consistently reflect either the convulsive activity seen or the protection from convulsive activity which combinations of these compounds cause.

4. It is concluded that the absolute level of GABA within the whole brain cannot be used as an index of the state of excitability of the brain.

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MICROANALYTICAL PROCEDURES FOR FLUOROMETRIC ASSAY OF BRAIN DOPA–5HTP DECARBOXYLASE, NOREPINEPHRINE AND SEROTONIN, AND A DETAILED MAPPING OF DECARBOXYLASE ACTIVITY IN BRAIN

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THE presence of the amines, norepinephrine (NE)† and serotonin (5HT), and of the enzymes, 3:4-dihydroxyphenylalanine (DOPA) and 5-hydroxytryptophan (5HTP) decarboxylases, in brain has aroused considerable conjecture regarding the role of the amines in the central nervous system. A detailed mapping of the decarboxylases and the amines in brain might outline the regions where the amines act and throw some light on their normal function.

Previous papers have described the fluorometric estimation of tissue NE (SHORE and OLIN, 1958) and 5HT (BOGDANSKI *et al.*, 1956) and the fluorometric (BERTLER and ROSENGREN, 1959*a*), or manometric (HOLTZ and WESTERMANN, 1956), estimation of 5HTP and DOPA decarboxylases. Although these methods are suitable for the estimation of these substances in relatively gross areas of the brain, they are not sensitive enough for their estimation in more precisely defined brain regions.

This paper describes modifications of the fluorometric procedures for NE and 5HT estimation whereby they can be assayed in amounts of brain containing as little as 40 μmg of NE or 200 μmg of 5HT. In addition, methods are described for measuring the activities of 5HTP and DOPA decarboxylases in 20 to 100 mg of brain tissue. With these methods the activity of DOPA decarboxylase in the cat brain has been mapped in considerable detail and has been related to the activity of 5HTP decarboxylase and to the levels of 5HT and NE.

ANALYTICAL METHODS

Preparation of brain tissue. Brains from cats killed with sodium pentobarbital were removed as rapidly as possible and dissected at 4°. The tissues were either analysed immediately, or frozen for analysis at a later time.

Estimation of NE in brain tissue.‡ The NE method (SHORE and OLIN, 1958) was adapted for analyses of small amounts of tissue as follows: Homogenize 50–200 mg of tissue in 0·01 N-HCl in a 15 ml glass-stoppered shaking tube in a total volume of 0·6 ml. Add 0·7 g of NaCl and 6 ml butanol. Shake for 1 hr on a shaking apparatus. Centrifuge the tube and transfer 5 ml of the butanol phase to a 25 ml glass-stoppered shaking tube containing 0·8 ml of 0·01 N-HCl and 10 ml of heptane. Shake for 15 min, centrifuge at low speed, and transfer 0·5 ml of the acid layer to a small test tube.

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† The abbreviations used are: NE, norepinephrine; 5HT, serotonin; DOPA, 3:4-dihydroxyphenylalanine; 5HTP, 5-hydroxytryptophan.

‡ Reagents and solvents were prepared as described by SHORE and OLIN (1958).

Add 0·2 ml acetate buffer, pH 5, and 0·02 ml of 0·1 N-iodine solution. Wait 6 min and add 0·04 ml of 0·05 N-sodium thiosulphate solution. After thorough mixing add 0·2 ml of NaOH–ascorbic acid solution,* Wait 30 min and transfer the solution to a quartz microcuvette, 20 mm × 3 mm × 3 mm (American Instrument Co.). Activate at 400 mμ and measure the resulting fluorescence at 510 mμ (Aminco-Bowman Spectrophotofluorometer, wavelengths uncorrected). Prepare standards by adding a known amount of NE to tissue homogenate and carrying the amine through the extraction procedure. Recoveries of NE (40–200 μmg) added to brain tissue ranged from 85–100 per cent.

Estimation of 5HT *in brain tissue.*‡ The 5HT method (BOGDANSKI *et al.*, 1956) was adapted for analysis of small amounts of tissue as follows: Homogenize 100–200 mg of tissue in 0·1 N-HCl in a 15 ml glass-stoppered shaking tube and dilute with water to a volume of 1·0 ml.† Adjust the pH to about 10 by the addition of anhydrous Na_2CO_3. Add 0·2 ml borate buffer, pH 10, 0·7 g NaCl, and 4 ml butanol. Shake for 30 min. Centrifuge the tube and transfer 3 ml of the butanol phase to a 15 ml glass-stoppered tube containing 6 ml of heptane and 0·5 ml of 0·1 N-HCl. Shake, centrifuge the tube, transfer 0·3 ml of the acid layer to a small test tube, and add 0·09 ml of concentrated HCl. Transfer the solution to a quartz microcuvette, activate at 300 mμ, and measure the resulting fluorescence at 540 mμ (wavelengths uncorrected). Prepare standards by adding known amounts of 5HT to tissue homogenate and carrying the amine through the extraction procedure. Recoveries of 5HT (200–500 μmg) added to aliquots of brain homogenates ranged from 90 to 100 per cent.

Estimation of DOPA decarboxylase in brain tissue. DOPA decarboxylase activity was determined by measuring fluorometrically the dopamine formed on anaerobic incubation of the tissue for 3 hr with an excess of DOPA. DOPA also fluoresces, but dopamine was separated from DOPA by extraction into butanol. Time-curves indicated that the rate of dopamine formed was linear for about 1 hr, and then declined somewhat over the next 2 hr. The amount of dopamine formed in 3 hr in various brain areas was proportional to the amount of enzyme. Enzyme activity was calculated as μg of dopamine formed/g of tissue per 3 hr.

Homogenize 20–100 mg of tissue in 0·25 M-sucrose and dilute to a volume of 0·6 ml. Transfer the homogenate to 20 ml beakers. Wash the homogenizer with 1·2 ml of water and add the washings to the homogenate. To the mixture, add 1·0 ml of 0·5 M-phosphate buffer, pH 6·9, and 0·1 ml of a 200 μg/ml pyridoxal phosphate solution. After preincubation of the mixture for 20 min in a metabolic shaker at 37° in an atmosphere of nitrogen, add 0·1 ml of a 2 mg/ml solution of L-DOPA and incubate for 3 hr.

To determine the dopamine formed, transfer the incubation mixture to a 60 ml glass-stoppered bottle containing 3 g of NaCl and 30 ml of butanol.§ Shake the bottle for 5 min, centrifuge, and transfer 25 ml of the butanol layer to a 125 ml glass-stoppered bottle containing 35 ml of heptane and 4 ml of 0·1 N-HCl. Shake the bottle for 5 min and centrifuge. Transfer 3 ml of the acid layer to a quartz cuvette. Activate at 280 mμ and measure the resulting fluorescence at 340 mμ (wavelengths uncorrected). Recoveries of dopamine (50 μg) added to the incubation mixture ranged from 80 to 95 per cent.

Estimation of 5HTP decarboxylase in brain tissue. The method for measurement of 5HTP decarboxylase activity (BOGDANSKI *et al.*, 1957) was adapted to the analysis of small amounts of tissue. Time curves indicated that the rate of 5HT formation was linear up to 2 hr. The rate of 5HT formation was proportional to the amount of brain homogenate. Enzyme activity is reported as μg of 5HT formed/g of tissue per hour.

Homogenize 30–120 mg of brain in 0·25 M-sucrose and dilute to a final volume of 1 ml. Transfer the homogenate to a 20 ml beaker. Wash the homogenizer with 2 ml of water and add the washings to the homogenate. To the mixture add 0·3 ml of M-phosphate buffer, pH 8·0 and 0·1 ml of a 200 μg/ml pyridoxal phosphate solution. After preincubation of the samples in a metabolic shaker for 20 min at 37° in an atmosphere of nitrogen, add 0·1 ml of a 10 mg/ml solution of DL-5HTP and incubate for 2 hr.

* Reagents and solvents were prepared as described by SHORE and OLIN (1958).

† A small plastic homogenizer was adapted to fit the shape of the 15 ml shaking tube.

‡ Reagents and solvents were prepared as described by BOGDANSKI *et al.* (1956).

§ The butanol was washed with 2 volumes of 0·01 N-HCl and repeatedly with water until the final pH of the wash was 7. The aqueous wash was left in the same bottle as the butanol and saturated with NaCl. It is important that the butanol be free of acid, otherwise it will extract DOPA as well as dopamine. A tissue homogenate containing only DOPA is extracted to correct for small amounts of DOPA carried through the procedure.

To determine the 5HT formed, transfer the incubation mixture to a 60 ml glass-stoppered bottle containing 3 g of NaCl, 3 ml of borate buffer, pH 10,* and 20 ml of butanol. Shake the bottle for 10 min and centrifuge. Transfer as much of the butanol layer as possible to a 50 ml glass-stoppered bottle. Wash the butanol layer twice by shaking with 10 ml of borate buffer, pH 10, to completely remove 5-hydroxytryptophan. Transfer 15 ml of the butanol layer to a 60 ml glass-stoppered bottle containing 30 ml of heptane and 3 ml of 0·1 N-HCl. Shake the bottle for 5 min and centrifuge. Transfer 2 ml of the acid layer to a quartz cuvette containing 0·6 ml of concentrated HCl. Activate at 300 mμ and measure the resulting fluorescence at 540 mμ (wavelengths uncorrected).

RESULTS

Distribution of decarboxylase in brain

The activity of DOPA decarboxylase varied widely from one part of the brain to another (Table 1). The activity of the enzyme is localized predominantly in grey matter and is low in white matter: fornix, medullary white matter, corpus callosum, pyramidal tract, and the optic tract. It is unevenly distributed in grey matter. For example, it is almost absent in the grey matter of cerebellum and is low in the neocortex.

The caudate nucleus has the highest activity of DOPA decarboxylase. Some parts of the rhinencephalon, the olfactory tubercle, septum, and amygdala, have substantial amounts of the enzyme, the olfactory tubercle being particularly high in activity. Other parts of the rhinencephalon, the pyriform cortex, the hippocampus, the cingulate cortex, the fornix, and the olfactory bulbs have low activity.

The diencephalon is for the most part rich in enzyme activity. The distribution of DOPA decarboxylase was established in various parts of the hypothalamus. The tissue was divided into anterior, posterior, and middle thirds, and into ventral and dorsal halves. The results show that the enzyme is fairly evenly distributed throughout the hypothalamus (Table 1). Considerable amounts of the enzyme are present in certain parts of thalamus, especially the intralaminar and basal thalamic regions, but the amounts in sensory thalamic nuclei are low.

In the midbrain, the decarboxylase level is high in the reticular formation, central grey matter, and the red nucleus. The enzyme is also concentrated in the reticular formation of the pons and the medulla.

A number of the specific classical sensory pathways as, for example, the visual and auditory, have low activity (Table 2). The DOPA decarboxylase activity in the olfactory bulb and the pulvinar is also low.

Comparison of the distribution of DOPA and 5HTP decarboxylase activities in a number of brain areas suggests that the ratio of the two activities is roughly the same in all areas of the brain. The constancy of this ratio became more obvious when the enzyme activities in nine different brain regions of the same animal were compared (Table 3). This finding lends support to the view that DOPA and 5HTP are decarboxylated by the same enzyme (WESTERMANN *et al.*, 1958; BERTLER and ROSENGREN, 1959*b*).

Distribution of decarboxylase and amines in brain

In most parts of the brain, the level of NE is roughly parallel to the activity of DOPA decarboxylase (Table 4). The caudate nucleus is a notable exception for it has a high amount of DOPA decarboxylase and has only a small level of NE. It is possible that the level of NE and the activity of the hydroxylase which converts

* Reagents and solvents were prepared as described by BOGDANSKI *et al.* (1956).

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TABLE 1.—DOPA DECARBOXYLASE ACTIVITY OF VARIOUS REGIONS IN CAT BRAIN

	DOPA decarboxylase activity μg of dopamine formed/g tissue per 3 hr.		DOPA decarboxylase activity μg of dopamine formed/g tissue per 3 hr.
Telencephalon		*Diencephalon (Cont).*	
Cerebrum grey and white matter (mean)	51	Hypothalamus (whole)	750
Neocortex (mean)	39	Anterior (third)	780
Visual cortex	51	Supra optic nucleus	780
Auditory cortex	69	Posterior (third)	732
Cruciate cortex	99	Middle (third)	792
Corpus callosum	120	Ventral (half)	600
Caudate nucleus	1260	Dorsal (half)	870
Rhinencephalon		Pituitary	159
Olfactory bulb	69	*Mesencephalon (midbrain)*	
Olfactory tubercle	930	Midbrain (whole)	648
Pyriform cortex	159	Reticular formation	810
Amygdala	291	Red nucleus	540
Cingulate cortex	51	Central grey matter	522
Hippocampus	81	Superior colliculus	201
Fornix	129	Inferior colliculus	201
Septum	420	*Metencephalon*	
Diencephalon		Cerebellum	21
Thalamus (whole)	400	Pons (mean)	300
Intralaminar and midline nuclei	660	Pontile reticular formation	381
Basal thalamic area	531	*Myelencephalon*	
Hypnogenic zone of Hess	261	Medulla (mean)	219
Pulvinar	159	White matter	120
Medial geniculate	129	Medullar reticular formation	351
Lateral geniculate	99	Cochlear nuclei	90

Each value represents the mean obtained from 2–6 animals.

TABLE 2.—DOPA DECARBOXYLASE ACTIVITY OF SOME SPECIFIC SENSORY PATHWAYS IN CAT BRAIN

	DOPA decarboxylase activity μg of dopamine formed/g tissue/3 hr.
Auditory pathway	
Cochlear nuclei	90
Inferior colliculus	201
Medial geniculate	129
Auditory cortex	69
Visual pathway	
Optic nerve	51
Superior colliculus	201
Lateral geniculate	99
Visual cortex	51
Pulvinar	159
Olfactory bulb	69

Each value represents the mean obtained from 3 cats.

TABLE 3.—RATIOS OF ACTIVITY OF DOPA DECARBOXYLASE TO 5HTP DECARBOXYLASE IN VARIOUS REGIONS OF CAT BRAIN

Area	Decarboxylase activity* DOPA	5HTP	Ratio
Caudate nucleus	1260	300	4·2
Hypothalamus	750	160	4·8
Basal thalamic area	660	135	4·8
Intralaminar and midline nuclei	400	80	5·1
Inferior colliculus	201	40	5·1
Medial geniculate	129	30	4·2
Hippocampus	99	23	4·2
Cochlear nucleus	90	20	4·5
Auditory cortex	69	15	4·5

Various tissues of 4 cats were pooled for estimation of DOPA and 5HTP decarboxylase.
* Units as in Table 4.

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dopamine to NE would show a closer relationship. In those parts of the brain in which the NE level is greater than 0·95 μg/gm, the DOPA decarboxylase activity is greater than 400 μg dopamine formed/g of tissue per 3 hr.

In general, brain areas high in NE content are also high in 5HT (Table 4). But in areas such as the amygdala, the pyriform cortex, caudate nucleus, and the hypnogenic

TABLE 4—ACTIVITIES OF DOPA AND 5HTP DECARBOXYLASE AND LEVELS OF NE AND 5HT IN VARIOUS REGIONS OF CAT BRAIN

Area	Concentration (μg/g) NE	5HT	Decarboxylase activity* DOPA	5HTP
Anterior hypothalamus	3·6	2·4	750	185
Posterior hypothalamus	2·0	2·5	896	175
Septum	1·5	2·0	420	—
Midbrain reticular formation	1·4	2·6	810	85
Central grey matter	1·2	1·6	519	—
Olfactory tubercle	0·95	2·0	930	264
Pontile reticular formation	0·97	0·89	383	85
Medial geniculate	0·50	—	129	30
Neocortex	0·49	0·69	39	—
Hypnogenic zone of Hess	0·45	1·8	261	45
Amygdala	0·30	1·6	291	35
Caudate nucleus	0·36	1·6	1260	300
Pyriform cortex	0·35	1·4	159	20
Auditory cortex	0·35	0·41	51	—
Cochlear nucleus	0·32	—	90	20
Inferior colliculus	0·26	0·76	201	40
Thalamus (whole)	0·17	0·43	231	50
Pons	0·25	0·33	300	40
Intralaminar and midline nuclei	0·40	0·56	400	80
Basal thalamic area	1·00	0·88	660	135
Cingulate cortex	0·35	0·41	51	—

Each value represents the mean obtained from 2–6 cats.
* Units for DOPA decarboxylase: μg dopamine formed/g per 3 hr. Units for 5HTP decarboxylase: μg 5HT formed/g per hr.

zone of Hess, the content of 5HT is high compared to that of NE. In these areas the 5HT content is much higher than would be expected on the basis of the decarboxylase activity.

DISCUSSION

The capacity of different parts of the brain to form dopamine is roughly proportional to the level of NE. An exception is the caudate nucleus, which is extremely high in DOPA decarboxylase activity and low in NE content; in this connection CARLSSON (1959) has found that the caudate nucleus contains an extremely high level of dopamine.

The constant ratio between the activities of DOPA and 5HTP decarboxylase in various brain areas is in agreement with the results of BERTLER and ROSENGREN (1959*b*) and supports the view that DOPA and 5HTP are decarboxylated by the same enzyme in brain as well as in other parts of the body (WESTERMANN *et al.*, 1958; YUWILER *et al.*, 1959). This is important from another point of view since the administration of an amine precursor such as 5HTP or DOPA might lead to decarboxylation at sites where the resulting amine is normally absent. This makes it difficult to interpret the pharmacological effects of injected precursors.

In recent years, it has been suggested that 5HT and NE function as neurohormones in the central nervous system. A detailed mapping of decarboxylase activity shows that it is present in high activity in the brain stem, the reticular formation, the hypothalamus, the basal and intralaminar portions of the thalamus, and in certain components of the rhinencephalon—in general, those areas which coordinate behavioural, autonomic, and somatic activity (HESS, 1954; MAGOUN, 1958). This suggests that NE and 5HT might be functionally associated with brain regions which control these activities. The level of the decarboxylase is low in other parts of the brain, including the white matter, cerebellum, and cortex. It is particularly significant that the activity is low in the nuclei of specific sensory pathways even when these are located near areas that have high amounts of the enzyme.

A recent development in neuroanatomy has been the mapping of extensive ascending and descending pathways between the diencephalon and certain limbic structures (NAUTA, 1958). The descending pathways include the amygdala, septum, pre-optic areas, hypothalamus, midbrain reticular formation, and central grey matter. These areas are also implicated in the ascending pathways which also connect with the reticular activating formation and through it with the basal thalamic region and intralaminar nuclei. Our data reveal that these areas, on the whole, contain considerable decarboxylase activity.

The striking similarity between the effects of chlorpromazine and of lowered ergotropic tone, and between the effects of reserpine and of increased trophotropic tone has raised the view that NE and 5HT regulate these opposing diencephalic systems postulated by HESS (BRODIE *et al.*, 1958). Because of the high precision and sensitivity of decarboxylase assay, we had anticipated that evidence for the two opposing systems might be shown by a differential distribution of 5HTP and DOPA decarboxylase. However, both 5HT and dopamine appear to be formed by the same enzyme. Furthermore, the distribution of 5HT and NE in brain does not show the differences that might have been expected from the functional differences described by HESS (1954). For example, though the anterior hypothalamus largely integrates trophotropic functions and the posterior hypothalamus ergotropic functions, both regions have

high levels of NE and 5HT. It is possible, however, that the overlap in distribution of the two amines is due to extensive reciprocal pathways between geographically close regions.

The decarboxylases and the amines are also present in parts of the central nervous system not studied by HESS, particularly the reticular formation, which receives afferent impulses from many sources and exerts a generalized activating effect on the brain (MAGOUN, 1958). It is possible that NE and 5HT regulate the activity of the reticular formation and its rostral projections into certain thalamic nuclei, the hypothalamus, olfactory tubercle, amygdala, and septal nuclei of the rhinencephalon (MCLEAN, 1959; NAUTA, 1958). Others have suggested that NE is a chemical mediator of the reticular formation (ROTHBALLER, 1956). In addition, recent studies show that the reticular formation can be inhibited as well as activated by peripheral stimuli (DELL *et al.*, 1954). The two amines might serve to control the opposing activating and inhibitory parts of the reticular formation and its rostral projections. Thus far, however, attempts to show that 5HT and NE exert antagonistic effects in the brain have yielded equivocal results.

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SUMMARY

Micro methods, presented for the assay of norepinephrine and serotonin, permit their fluorometric assay in amounts of nervous tissue containing as little as 40 μmg of NE and 200 μmg of 5 HT.

Fluorometric methods are presented for the measurement of DOPA and 5HTP decarboxylase in 20–100 mg of brain tissue.

The finding that the ratio of DOPA to 5HTP decarboxylase activity is essentially constant in various parts of the brain indicates that both amino acids are decarboxylated by the same enzyme.

A detailed mapping of the distribution of DOPA–5HTP decarboxylase shows that most of the enzyme is localized in those brain regions that coordinate behavioural, autonomic, and somatic activity; that is, in the reticular formation, the hypothalamus, the basal and intralaminar parts of the thalamus, and in certain portions of the rhinencephalon.

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THE DISTRIBUTION OF THE ARYLAMINO GROUP IN THE BRAIN OF THE ALBINO RAT FOLLOWING ADMINISTRATION OF *p*-AMINOBENZOIC ACID

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IT IS well recognised that many substances fail to pass rapidly from the blood to the brain *in vivo*. In many such cases the failure to penetrate into brain tissue seems to be attributable to some peculiar feature of the cerebral capillary walls or adjacent glial cells, forming the postulated 'blood–brain barrier' (for references see KING, 1938; LAJTHA, 1957). Probably no substance is known which passes the blood–brain barrier easily but is completely stopped by the nerve cell membranes. This accounts for the uncertainty about the extent of the 'extra-cellular' compartment in the mammalian brain. Chloride ion has been generally considered to be the most reliable indicator of 'extracellular' space in the brain (MANERY, 1954), but probably the space so determined is too large. Recently, on the basis of studies with the electron microscope, it has been stated (GERSCHENFELD *et al.*, 1959) that no true extracellular space exists in brain tissue.

The observations upon rat brain recorded below show that the arylamino group derived from *p*-aminobenzoic acid (PAB), administered as the sodium salt, traverses the blood–brain barrier readily, but is confined to a space much less than that which corresponds to the total water content of the tissues. The magnitude and distribution in different parts of the brain, of the spaces so determined, together with the corrections to be applied and the possible relationship to extracellular space, are discussed.

METHODS

Wistar Albino rats of 160–180 g weight were used. *p*-Aminobenzoic acid (300 mg) was administered by stomach tube as the sodium salt in 5 ml water. *p*-Aminobenzenesulphonamide (500 mg) was given as a suspension in 5 ml water with gum tragacanth.

At intervals which varied from 1·5 to 4 hours after administration of the test substances, the animals were killed by stunning and decapitation, and the blood was collected over sodium oxalate.

The brains were removed, and samples of approximately 100 mg wet weight, taken from definite areas, were transferred rapidly to tared papers, weighed on a torsion balance, and dropped into 5 ml of 5% trichloroacetic acid. After hardening for a few hours, the tissue was broken up with a glass rod and left in contact with the trichloroacetic acid overnight.

The six areas of brain selected were arbitrarily delimited on gross inspection. They included frontal, parietal and occipital cerebral cortex, the major diencephalic nuclei, the pons and upper medulla, and the cerebellum.

Free *p*-aminobenzoate and sulphanilamide (where used) were estimated photometrically, in portions of the original trichloroacetic acid extracts after filtration or centrifugation, by the method of BRATTON and MARSHALL (1939). Measurements were made on the Zeiss Pulfrich Step Photometer using filter S53. The blood content of the rat brain was estimated by a slight modification of the acid haematin method described by TINGEY (1938). No attempt was made in the present study to estimate separately acetylated or other conjugated forms of the arylamino compounds used. It was found

that after administration of *p*-aminohippurate the blood level of this compound two hours later was negligible, so that it was considered unlikely to interfere. Confirmation of this was obtained by chromatography, carried out on Whatman no. 1 strips by the ascending method with *n*-butanol–acetic acid–water (4:1:1). After drying, the strips were exposed to nitrous fumes (7–8 min), left in the air for 5 min, and sprayed with a mixture of equal parts of 1% ammonium sulphamate and the Bratton–Marshall colour-developing reagent, followed by hydrochloric acid fumes. Arylamino compounds showed as red-violet spots. It was clear that the material found in the plasma of PAB-treated rats was mainly *p*-aminobenzoate (R_F 0·76–0·78) whilst only the faintest trace of material, or none at all in some strips, was found to correspond with the *p*-aminohippuric acid control spot (R_F 0·49–0·51). However, there was in the plasma a small proportion of chromogenic material running with R_F 0·22–0·24. This material was not identified, but it was estimated by comparison with control spots that it might contribute 2–5 per cent of the total colour measured in plasma samples. For convenience, the material estimated in samples taken from rats treated with *p*-aminobenzoate has been referred to as such, although the method used was not strictly specific for this compound.

CALCULATION

The apparent space occupied by the test substance, on the assumption that it was distributed at equal concentrations in the available water phases of brain and blood plasma respectively, was calculated as a percentage of the wet weight of the tissue samples by the formula:

$$93 \times \frac{w_p \cdot e_s}{e_p \cdot w_s}$$

where e_s and e_p are extinction values at 530 mμ from weights (mg) w_s and w_p of tissue sample and plasma respectively. The factor 93 is derived from the mean percentage of water found in oxalated plasma. The apparent space so calculated would require correction should the distribution of test substance in the water phases of blood and brain be otherwise than at equal concentrations.

RESULTS

As shown in Table 1, the apparent spaces calculated for *p*-aminobenzoate were considerably less than those which correspond to the total water content, in all parts of the brain studied. They were, however, considerably larger than the total plasma content of whole rat brain estimated as 60 per cent of the total blood content (3·4 ml/100 g wet tissue) or just over 2 per cent of the wet weight. The value for the blood content of rat brain is much higher than that quoted for rabbit brain (0·6 per cent) by BRIERLY (1956) but less than the figure of 4·9 per cent given by GELL, POLIS and BAILEY (1955) for rat brain.

It is clear that the amounts of *p*-aminobenzoate found in the tissue were greater than could be accounted for by the blood content, and less than would be expected if most of the tissue water held the substance at a concentration comparable with that in plasma. This limited penetration did not seem to be markedly time-dependent, since figures obtained 4 hr after administration of *p*-aminobenzoate were not very different from those after 2 hr, though plasma levels had fallen in the interval from 50 mg per cent to about 30 mg per cent.

The region of the pons and medulla, which contains a relatively higher proportion of white matter than the other portions of the brain studied, showed the lowest percentage of diffusion space available for *p*-aminobenzoate.

Table 2 shows for comparison the results of similar experiments carried out with *p*-aminobenzenesulphonamide, a neutral substance with a molecule of approximately equal size. In this case the figures indicate that a larger proportion of the tissue water is penetrated by sulphanilamide than by *p*-aminobenzoate. The more limited

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penetration of the latter substance into the brain tissue was therefore not attributable to inadequate duration of the experiments since this limitation might have been expected to apply equally to sulphanilamide.

Asymmetrical distribution of p-*aminobenzoate.* The presence of non-diffusible ions at different concentrations on either side of a membrane will in general lead to

TABLE 1.—APPARENT *p*-AMINOBENZOATE SPACE IN RAT BRAINS EXPRESSED AS PERCENTAGE OF WET WEIGHT

Duration of experiment	A	B	C	D	E	F
(hr)						
1·75*	15	16·5	18·3	16·7	10·9	9·0
2	17·6	16·1	18·4	16·9	14·4	10·0
3·5	19·2	20·3	20·7	16·2	12·9	9·2
2*	19·6	20·8	21·0	17·2	17·1	9·2
2·25	15·6	17·0	16·8	13·8	12·8	8·6
3	15·2	16·3	16·0	14·8	12·2	10·2
4	16·5	19·3	19·3	14·9	15·2	8·8

A = Frontal cortex
B = Parietal
C = Occipital
D = Cerebellum
E = Diencephalic nuclei
F = Pons–medulla.

* Substance administered in 5 ml of 30% ethanol.

TABLE 2.—APPARENT SULPHANILAMIDE SPACE IN RAT BRAINS EXPRESSED AS PERCENTAGE OF WET WEIGHT

Duration of experiment	A	B	C	D	E	F
(hr)						
2	61	55	58	48	46	29
2·5*	60	63	68	54	62	42
2·5*	59·4	69·2	65·1	41·8	45	26
2·25	57	51·9	59·7	66·7	49·4	37·3
2	50·5	49·1	56·9	48·9	45·4	27·8
2·25*	56·7	60·2	66·0	55·9	52·9	36·3

A = Frontal cortex
B = Parietal
C = Occipital
D = Cerebellum
E = Diencephalic nuclei
F = Pons–medulla

* Substance administered in 5 ml of 30% ethanol.

an asymmetric distribution at equilibrium of molecules carrying charges and capable of passing freely through the membrane. The conditions will not necessarily correspond to those of the classical Donnan equilibrium, as was shown when *p*-aminobenzoate was allowed to distribute itself across a cellophane membrane between human plasma and a buffered isotonic saline at pH 7·4. The experiment was conducted at 4° over a three-day period. The saline was Ringer-Locke solution to which was added 0·15 M-phosphate buffer (pH 7·4) in the proportion of 5 ml of phosphate

to 100 ml of saline. Equilibrium was reached after the second day, when the ratio:

$$\frac{\textit{Concentration of PAB in water on plasma side}}{\textit{Concentration in water on saline side}}$$

was found to be 1·12. The concentrations of *p*-aminobenzoate used were in the same range as those encountered in the rat experiments, but otherwise, notably in temperature, the conditions of this diffusion experiment differed from those *in vivo*. Nevertheless the figure obtained presumably gives some guidance on the maximal correction which would have to be applied for an asymmetrical distribution resulting from the concentration of protein in plasma, if a simple diffusion process is assumed to occur at the capillary walls.

DISCUSSION

The determination of the 'space' occupied by a chemical substance in a tissue or organ involves in itself some troublesome assumptions, and its interpretation even more. The chloride space in brain has been widely regarded as a measure of the extracellular space, but as MANERY (1954) pointed out, the chloride space in brain may be calculated with reference to either blood plasma or cerebrospinal fluid levels. This possibly accounts for the widely different values for mammalian brain quoted by different authors, e.g. 31 per cent for rabbit brain, given by DAVSON (1955) and 22 per cent for rat brain calculated by LAJTHA (1957). On the basis of resting potentials of single cells, ECCLES (1957) has calculated that about one fifteenth of the brain chloride is intracellular, so that LAJTHA's figure quoted above would indicate for whole rat brain an extracellular space of 21 per cent.

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TABLE 3.—*p*-AMINOBENZOATE SPACE AND TOTAL WATER CONTENT OF RAT BRAIN AS PERCENTAGES OF WET WEIGHT

	Cerebral cortex	Cerebellum	Diencephalon	Pons and medulla
PAB space (apparent)	17·9	15·8	13·6	9·3
PAB space (corrected)	20·0	17·7	15·2	10·4
Water content	79·1	76·1	76·1	72·2
Sulphanilamide space	59·3	52·5	50·1	33·0
Ratio PAB space/sulphanilamide space	0·34	0·34	0·31	0·32

It is noteworthy that in most parts of the brain except the brain stem, the *p*-aminobenzoate space found in the present series of experiments turns out to be slightly less than this figure, and approximates to it (Table 3) if a correction is made on the assumption that the interstitial fluid phase has a very low protein content.

The general similarity between the blood–brain and blood–CSF barriers gives some support to such assumptions, but recent observations (GERSCHENFELD *et al.*, 1959) place this matter in doubt. If these workers' findings with the electron microscope are accepted, the function of extracellular fluid in brain is taken over by specialised extensions of the glial cytoplasm. There is in fact, according to them, no true extracellular space as usually understood. A cytoplasmic phase, involved in transport of material between blood and nerve cells, could contain appreciable concentrations of protein and other non-diffusible ions which introduce uncertainty into calculations of the space.

The principle of the limited rapid initial penetration of a suitable substance into tissue has often been used for the measurement of extracellular space, e.g. by ALLEN (1955) who studied cerebral cortex slices *in vitro* with the aid of inulin and ferrocyanide, and found values of 14·5 per cent and 17 per cent respectively for the spaces so determined. The present study shows that *p*-aminobenzoate indicates *in vivo* a well-defined space of similar size which appears to be functionally equivalent to extracellular space in other tissues, if the above-mentioned criterion is considered valid.

The size of the *p*-aminobenzoate space in the pons–medulla region was much less than elsewhere, and out of proportion with the rather lower water content of this part of the brain. The ratio between this space and the sulphanilamide space was however much the same as in other parts, namely 0·31–0·34.

In some experiments 30% (v/v) ethyl alcohol was used in place of water as the vehicle for administration of *p*-aminobenzoate. Since it was desired to use the space-indicating property of *p*-aminobenzoate in electrolyte studies on alcohol-treated rats, it was necessary to know whether or not alcohol would, through permeability changes or otherwise, grossly alter the distribution of the test substance in brain. This did not appear to be the case.

SUMMARY

The apparent space occupied in six regions of the rat brain by arylamino-compounds after administration of *p*-aminobenzoate, has been measured by simultaneous estimations in blood and tissue samples. Throughout most of the brain, the *p*-aminobenzoate space was found to be close to the estimates of extracellular space made by previous workers for whole brain or brain slices. In the pons–medulla region, this space was relatively much smaller than elsewhere, even after allowance for the lower water content.

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Note: Since the above paper was written, there has been evidence of continuing differences of view between interested workers on the question of the existence of extracellular space in the mammalian brain. Thus EDSTRÖM (1960) considers that his work with radiochromium supports the idea that extracellular space is virtually non-existent in the rabbit brain. DAVSON and SPAZIANI (1959) on the other hand have clearly indicated their view that low values for this space, obtained *in vivo*, reflect the difficulty of finding suitable substances that penetrate the blood–brain barrier.

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ACTIVITY OF MONOAMINE OXIDASE IN RELATION TO THE 5-HYDROXYTRYPTAMINE AND NOREPINEPHRINE CONTENT OF THE RAT BRAIN*

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THE enzyme monoamine oxidase (MAO) is probably, at least in part, responsible for the metabolism of biogenic monoamines (e.g. tryptamine, 5-hydroxytryptamine (5HT), tyramine, catecholamines, O-methylcatecholamines) in the brain (see PLETSCHER *et al.*, 1960). The physiological function of the enzyme is, however, not clearly established. Thus, the fundamental question has not been answered whether or not MAO regulates the physiological content of monoamines in animal tissues. In the present paper this problem was investigated by comparing the activity of MAO and the level of monoamines in rat brain after injection of iproniazid, a long acting inhibitor of the enzyme in brain (ZELLER *et al.*, 1952, 1955; DAVISON *et al.*, 1957; DAVISON, 1958; HESS *et al.*, 1958).

EXPERIMENTAL

Pretreatment of animals. Female Wistar rats, fasted for 16 hr and weighing 60–80 g, received single injections of various doses of iproniazid (1-*iso*nicotinyl-2-*iso*propylhydrazide, Marsilid®) 16 hr before decapitation.

Tissue preparation for enzymic assay. The brains with the medulla oblongata of 8–10 animals were washed, pooled and homogenized (Potter–Elvehjem homogenizer with Teflon pestle, clearance 0·1–0·15 mm) in 4 vol of 0·25 M-sucrose (Baker). After slow centrifugation (30 min at about 500 **g**) some of the supernatant was centrifuged sharply (30 min at about 30,000 **g**). The particulate fraction including the fluffy layer was washed with 0·067 M-sodium potassium phosphate buffer, pH 7·2–7·3 (Merck or Baker), recentrifuged (20 min at 12,000 **g**) and the sediment suspended again in fresh phosphate buffer. All operations were performed at 2–4°. The particulate fraction was used because of a relatively low blank oxygen uptake and a constant O/NH_3 ratio in the absence of cyanide and semicarbazide.

MAO *activity.* Oxygen uptake was measured at 37·5° in Warburg manometers (shaking frequency 120/min) with 10% KOH and filter paper in the centre well, during 60–120 min after tipping the substrate (tyramine HCl, Roche, final concentration 0·009 M). During this period oxygen uptake was linear. The gas phase consisted of oxygen (maximal flushing at room temperature for 1 min) or air. The flasks were allowed to equilibrate on the bath for 15 min before adding the substrate and contained enzyme quantities corresponding to 0·6 g fresh tissue. Three portions of the particulate fraction of each brain pool were incubated with and 2 portions without substrate. MAO activity is expressed as μl O_2/hr per g original wet tissue corrected for oxygen uptake without substrate.

Ammonia produced during the incubation period was estimated in microdiffusion units (CONWAY, 1957) using a portion of rapidly chilled particulate fraction from the Warburg vessels. The results are expressed as μl NH_3/hr per g original fresh tissue corrected for blanks incubated anaerobically with substrate. Cyanide and semicarbazide (CREASEY, 1956) were not added to the incubation medium because these compounds may increase MAO activity *in vitro* (DAVISON, 1957) as well as after pretreatment with MAO inhibitors *in vivo* (unpublished results). The O/NH_3 quotient of the particulate fraction was 1·6–1·8 and remained fairly constant.

* The results were partly reported in a preliminary form at the Meeting of the Swiss Society for Biochemistry, Zurich, May 30, 1959 (GEY and PLETSCHER, 1959).

Estimation of 5HT *and norepinephrine* was carried out by spectrophotofluorometric procedures. Two brains were pooled for the 5HT assay according to BOGDANSKI *et al.* (1956). No difference was found whether the brains of normal and iproniazid pretreated animals were placed in 0·1 N-HCl of room temperature or in liquid nitrogen followed by homogenization in 0·1 N-HCl at about +4°.

For the determination of norepinephrine a slightly modified procedure of BERTLER *et al.* (1958) was used.

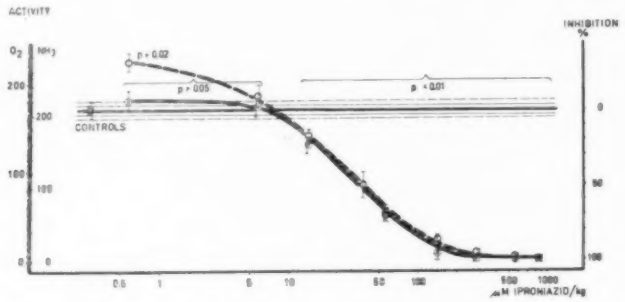

FIG. 1.—Monoamine oxidase of rat brain as measured manometrically under oxygen 16 hr after i.p. injection of iproniazid.

Each point represents mean activities ± mean error of 2–7 (mostly 4–6) animal brains. The iproniazid dose (±fiducial limits for 95 per cent probability) for 50 per cent inhibition was calculated by regression analysis to be 36 (29–44) μmoles/kg for oxygen and 32 (26–41) μmoles/kg for ammonia. Dotted line: oxygen consumption; solid line: ammonia formation.

Abscissa—μmoles iproniazid/kg

Ordinates—Activity μl/g per hr; Inhibition %

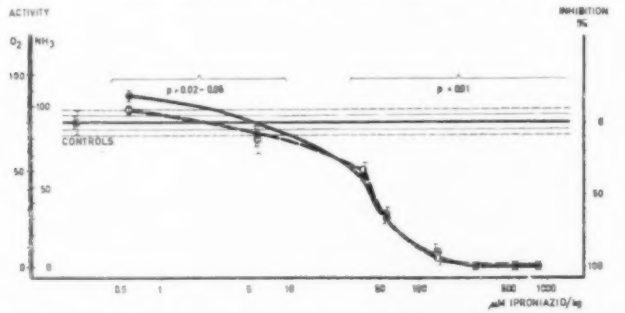

FIG. 2.—Monoamine oxidase of rat brain as measured manometrically under air 16 hours after i.p. injection of iproniazid.

Each point represents mean activities ± mean error of 2 animal brains.

Dotted line: oxygen consumption; solid line: ammonia formation.

Abscissa—μmoles iproniazid/kg

Ordinates—Activity μl/g per hr; Inhibition %

RESULTS

The influence of iproniazid 16 hr after i.p. injection on MAO activity as well as on 5HT content of rat brain is shown in Figs. 1–3. After 30–40 μmoles iproniazid/kg 50 per cent inhibition of MAO was reached. This iproniazid dose did not increase 5HT significantly ($P < 0.05$). The 5HT content started to rise significantly ($P > 0.01$) after administration of 112 μmoles iproniazid/kg (20 mg/kg). At this dose MAO was inhibited by about 85–90 per cent. With larger amounts of iproniazid the 5HT levels rose gradually: 560 μmoles/kg (100 mg/kg) doubled the 5HT content and inhibited MAO by 99–100 per cent. Excessive doses of iproniazid (up to 2,240 μmoles/kg = 400 mg/kg) did not elevate the 5HT content further.

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The degree of MAO inhibition by a given iproniazid dose did not differ significantly whether the particulate fraction was incubated under oxygen (Fig. 1) or air (Fig. 2).

The content of norepinephrine in rat brain behaved similarly to that of 5HT (Fig. 4). Iproniazid in doses of 140 μmoles/kg and more caused significant ($P < 0{\cdot}01$) progressive increase of norepinephrine which was, however, less marked than that of 5HT.

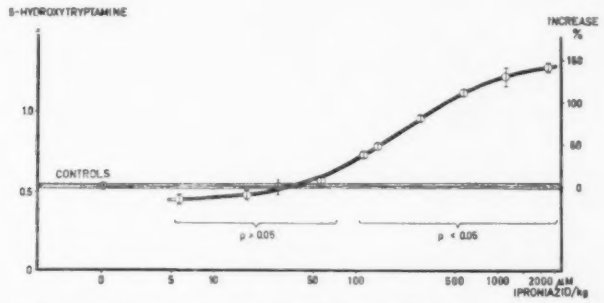

FIG. 3.—5-Hydroxytryptamine of rat brain 16 hours after i.p. injection of iproniazid. The points represent mean values and mean error of 48 control determinations and of 6–41 determinations for each dose of iproniazid.
Abscissa—μmoles iproniazid/kg
Ordinates—5-Hydroxytryptamine μg/g fresh weight; Increase %

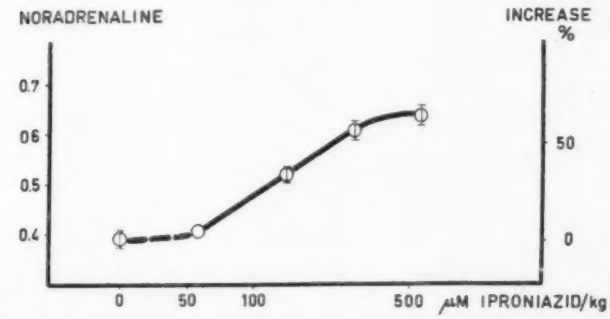

FIG. 4.—Norepinephrine of rat brain 16 hours after i.p. injection of iproniazid.
Series of 8 rat brains were homogenized in perchloric acid at +4° under CO_2 in a Servall-Omni mixer; norepinephrine was determined in 2–3 portions of each pool according to BERTLER *et al.* (1958). The points represent mean values ± mean error of 4 pools (one pool for the lowest iproniazid dose).
Abscissa—μmoles iproniazid/kg
Ordinates—Noradrenaline μg/g fresh weight; Increase %

DISCUSSION

According to the various dose/response curves an increase of the 5HT and norepinephrine content of rat brain occurs only when the degree of MAO inhibition amounts to 85 per cent or more. A similar relationship has been found in mouse brain comparing 5HT content and MAO activity in relation to time after injection of β-phenylethyl hydrazine, another long acting MAO inhibitor (CHESSIN *et al.*,

1959). Thus it can be assumed that MAO of brain is present in excess because a high degree of MAO inhibition is needed in order to produce an accumulation of monoamines.

This hypothesis implies that inhibition of MAO as measured *in vitro* reflects the situation *in vivo*. Several findings agree with such a supposition:

(a) MAO of slices and various cell fractions of brain homogenates is inhibited by iproniazid *in vitro* to about the same extent (ZELLER *et al.*, 1955). Thus, the degree of MAO inhibition seems not to depend on the method of enzyme preparation or on any unknown cofactors. The same findings indicate that dilution of the enzyme *in vitro* probably does not influence the degree of MAO inhibition. This is also to be expected because MAO inhibition by iproniazid is not easily reversed by washing procedures or dialysis (ZELLER *et al.*, 1955; DAVISON, 1957).

(b) Oxygen pressure, which is a limiting factor for MAO activity *in vitro* (PHILPOT, 1937; KOHN, 1937; BERNHEIM and BERNHEIM, 1938) does not influence the degree of MAO inhibition by iproniazid (Figs. 1 and 2). The amount of oxygen dissolved in the incubation medium under the experimental condition is equivalent to 4 and 18 mm Hg respectively (air and oxygen incubation) according to the Bunsen coefficient. This corresponds to the oxygen tension of mammalian brain *in vivo* which varies between 2 and 25 mm Hg depending on the distance from blood vessels (DAVIES and BRONK, 1957).

(c) The influence of substrate concentration has also to be considered. The monoamine content in the brain *in vivo* is probably in such a low range that MAO activity depends on substrate concentration. This is not the case *in vitro* because excess of substrate has been used for technical reasons. It does not, however, seem very likely that a decrease of substrate can influence the degree of inhibition since diminution of the other reacting partner, i.e. oxygen, is not able to do so.

The presence of measurable amounts of 5HT in brain does not contradict the hypothesis of the presence of MAO in excess. It has been shown that the amine is mainly stored in granules (GIARMAN and SCHANBERG, 1958; WALASZEK and ABOOD, 1959) and thus possibly protected from enzymic transformation (PLETSCHER *et al.*, 1956).

Among physiological compounds hitherto investigated only weak MAO inhibitors have been found. The most active, i.e. cortisone (SCHWEPPE *et al.*, 1959) and thyroxine (ZILE and LARDY, 1959; ZILE, 1960) in high doses, depressed the enzyme activity by 30 and 50 per cent respectively. This degree of inhibition does not influence the amine content according to the presented findings. Thus there is at present no indication that MAO activity influences monoamine content of brain under physiological conditions. The great excess of MAO rather suggests that the function of the enzyme consists in rapid inactivation of monoamines as soon as they are available, e.g. after release from storage depots.

SUMMARY

The influence of increasing doses of the monoamine oxidase (MAO) inhibitor iproniazid (1-*iso*nicotinyl-2-*iso*propylhydrazide, Marsilid®) on monoamine oxidase activity as well as on the content of endogenous 5-hydroxytryptamine and norepinephrine in rat brains was measured.

A significant rise of the amines occurred only when MAO was inhibited by at

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least 85–90 per cent. The degree of MAO inhibition by iproniazid was not influenced by the oxygen tension in the incubation flasks.

The results indicate that MAO is probably present in excess in rat brain and that the function of the enzyme presumably consists in rapid inactivation of free monoamines rather than in regulating the content of total monoamines.

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APPARATUS FOR APPLYING ELECTRICAL PULSES TO ISOLATED CEREBRAL TISSUES DURING METABOLIC STUDIES

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SINCE the description by McILWAIN (1951*a*), AYRES and McILWAIN (1953), and HEALD and McILWAIN (1956) of apparatus for producing electrical pulses and applying them to isolated tissues, the following apparatus and techniques have been devised and are described below. (1) A simplified generator of condenser pulses of alternating polarity. (2) An apparatus for interrupting a continuous series of such pulses so that they can be applied for successive brief, timed, periods. (3) Connections to electrode vessels. (4) Electrodes for localized application of pulses. (5) Quick-transfer apparatus for slices of various sizes.

DESCRIPTION OF APPARATUS

Pulse generator

The pulse generator and monitor earlier described (AYRES and McILWAIN, 1953) incorporated a simple circuit (McILWAIN, 1951*a*) in which condensers were charged and discharged by a mechanical relay; the d.c. supply to the condensers was derived from a.c. mains. The original instruments continued in almost daily use for 4 years, when replacement of the cathode ray tube and of some adjustable resistors was necessary. Subsequent apparatus for this purpose has been made with the pulse generator distinct from the monitor. Description of the present, more versatile pulse generator is given below; in place of the specially made monitor, a commercial oscilloscope is now used, the Cossor model 1049 being very suitable though more elaborate than is needed for this work alone.

Condenser-pulse generator. This modified instrument was designed and constructed by Mr. J. W. WESTHENRY. The circuit and details of components are given in Fig. 1. The different currents needed by the generator are supplied by one transformer which is switched on and off by a double-pole switch S_1 in the primary winding; this has tappings so that mains a.c. at 110 to 240 v can be used. One secondary winding supplies current at about 45 v, which is rectified and smoothed by condenser C_1 and resistance R_1. Across C_1 is the variable resistance R_2 which controls the output voltage (from 0 to approximately 30 v).

Another secondary winding from the transformer supplies alternating current at 7 v and 50 cycle/sec to the magnets of the relays which charge and discharge condensers C_3 to C_{11} to give the output pulses. The generator has two similar relay systems, of which one is shown in the diagram; the second reproduces all the components drawn to the right of the rectifier. The relays are brought into action

by individual 4-pole switches S_2 (A and B); S_4 (A and B), similarly connected, is not shown in Fig. 1. Operation of the relays is indicated by lamps L_2 and a corresponding L_3.

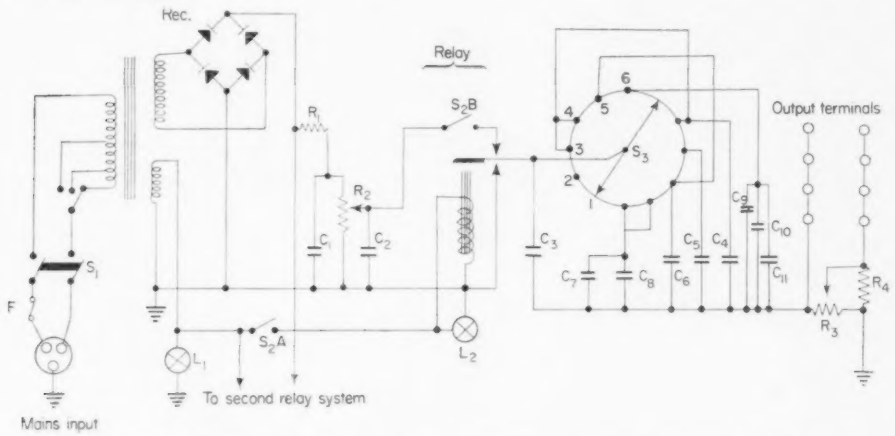

FIG. 1.—Circuit diagram of pulse generator. The components are as follows. C_1 and C_2, 500 μF; C_3, 1 μF; C_4 and C_8, 2 μF; C_5, 4 μF; C_6, C_7, C_9, C_{10}, and C_{11}, 8 μF. Fuse F, 2 A. Indicator Lamps: L_1 for mains and L_2 for the first relay system. Rec: metal rectifier. Relay: Carpenter type 3E9. Resistors: R_1, 270 Ω; R_2, continuously variable, controlling pulse amplitude; 1000 Ω. R_2, continuously variable, controlling pulse duration; 500 Ω. R_4, 10 Ω. Switches: S_1, mains; S_2, double pole on-off switch for the first relay system (S_1A, activating current; S_1B, input current). S_3, 6-way switch. Second output and other details: see text.

One of the fixed contacts of the first relay is connected to the slider of R_2 and the other to earth. The moving contact of the relay in one contact position, connects the output through R_1, to a series of condensers, and in the other position connects these condensers to earth. Each time connection is made, the current passing is limited by the capacity of the condensers, which is adjustable in steps by the 6-way switch S_3, giving the coarse adjustment of pulse duration. In its different positions the switch incorporates capacities of 1, 3, 7, 11, 19 and 35 μF. The fine adjustment of pulse-duration is given by the variable resistance R_3, which is in parallel with the output terminals and in series with a small permanent padding resistance R_4.

Reasons for choice of most of the components are given by AYRES and MCILWAIN (1953) and the performance of the generator is similar to the instrument then described. Stimulation by condenser pulses has also been used by KRATZING (1956) and by WALLGREN and KULONEN (1960). In the present generator two independent relays and groups of output terminals are arranged so that pulses of different characteristics can be compared in the same experiment. Also two independent experiments can be supplied from an instrument. Each relay will supply at the same time, up to six of the electrodes described below.

Pulse interruptor and timing circuit

This circuit was designed and the instrument constructed by Mr. P. J. W. AYRES. It receives a continuous series of pulses at 100/sec, and interrupts the series so that the output consists of chosen groups of pulses, at chosen intervals. The number of pulses in each group, and the duration of the intervals, are shown on dekatron

indicator valves. Application of the instrument to metabolic studies has been described by McILWAIN (1954), LEWIS and McILWAIN (1954), WOLLENBERGER (1955) and McILWAIN and GREENGARD (1957). Its operation is as follows (see Fig. 2 and Table 1).

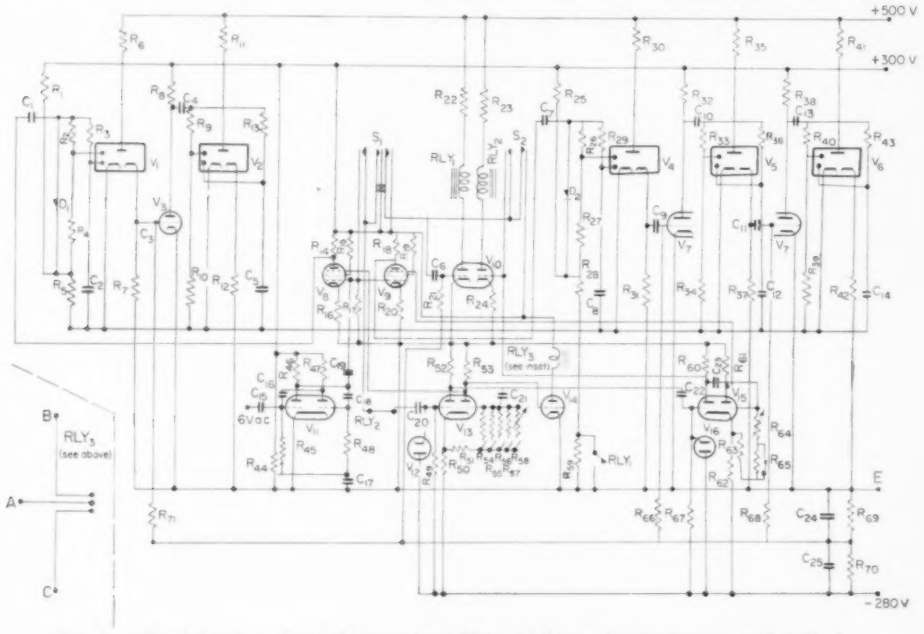

FIG. 2.—Circuit diagram of pulse interruptor and interval timer; the components are described in Table 1. The input at terminal *A* (see inset) is delivered either to terminal *B* or to *C*, according to the position of relay RLY3 (see main diagram).

A multi-vibrator valve, V.11, was set to a frequency of 100 pulses/sec. Alternating current at 6 v, 50 cycles was applied to grid 1 of valve V.11 in order to lock the multi-vibrator at twice the frequency of the mains supply. The output of this multi-vibrator was fed to the grids of valves V.8 and V.9 which were used as gating valves and delivered output pulses to either the pulse-indicating dekatrons V.1 and V.2, or to the interval-timing dekatrons, V.4, V.5 and V.6. A flip-flop trigger valve, V.13, operated gate valve V.8 and a pulse from the multi-vibrator was used to initiate the opening of V.8. Pulses were then recorded on dekatrons V.1 and V.2 as long as the trigger circuit remained operating, this interval being controlled by resistors R.54, 55, 56, 57 and 58. A push-button pre-set switch was used to select each resistor in turn and the values of R.54, 55, 56 and 57 were chosen to give time intervals for 2, 4, 6 and 8 pulses. These resistors were adjusted to appropriate values by filing ordinary carbon resistors until the correct timing interval was obtained. R.58 allowed the repetitive delivery of any number of pulses from 9 to 99.

While this trigger circuit was in the open position the grid of V.14 was held at earth potential and relay RLY 3 allowed the continuous series of pulses coming into the instrument at terminal *A*, to be delivered to terminal *B*. When this trigger circuit returned to its quiescent state V.14 and V.8 were cut off. Pulses applied at *A* were now delivered to terminal *C* and dekatrons V.1 and V.2 ceased to count, while a

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TABLE 1.—VALUES OF COMPONENTS OF PULSE-INTERRUPTOR
The components are numbered according to Fig. 2

Description of component *Resistances*	Value (Megohms)	Description of component *Condensers*	Value (μF)
R.1, 25, 70	0·270	C.1, 7, 17	0·1
R.5, 28	0·027	C.2, 3, 8, 9, 11	0·01
R.2, 3, 4, 26, 27, 29, 46, 47	0·068	C.4, 10, 13, 21, 23	0·25
R.6, 11, 17, 21, 30, 35, 41	1·2	C.5, 12, 14	0·02
R.7, 31	0·330	C.6	0·001
R.8, 32, 38	0·039	C.15, 19	0·05
R.9, 14, 18, 33, 40, 51, 63, 59	0·1	C.16, 18	0·0075
R.10, 13, 34, 36, 39, 43	0·15	C.20, 22	75×10^{-6}
R.66, 68, 71	0·47	C.24 (50 v, D.C., Electrolytic)	50
R.12, 15, 19, 37, 42	0·22	C.25 (350 v, D.C., Electrolytic)	8
R.16, 20	0·002		
R.22, 23	0·056		
R.24, 50, 62	0·005	*Valves*	(Type)
R.45, 48	2	V.1, 2, 4, 5, 6	GCl OA Dekatron
R.44 (potentiometer)	0·2	V.3, 14	6J5
R.49, 67	0·020	V.7, 10, 11, 13, 15	6SN7
R.52, 60, 61	0·12	V.8, 9	V.R.116
R.53, 51	0·025	V.12, 16	EA.50
R.54, 55, 56, 57 (see text)	—	D.1.2	Germanium diodes
R.58 (potentiometer)	1		
R.64 (potentiometer)	5	*Relays*	(Type)
R.65	5	RLY.1, 2	Siemens high speed relays
R.69	0·015	RLY.3	Carpenter Relay 3E.26

positive pulse was delivered to grid 1 of V.15 from anode 1 of V.13. V.15 is a flip-flop trigger circuit, which when operating opened gate V.9 and allowed pulses from the multi-vibrator to pass to dekatrons V.4, 5 and 6. These dekatrons now started to count the interval. In order that V.13 should not be triggered by the next incoming pulse from the multi-vibrator to its grid, anode 1 of V.15 took the grid of V.10, negative with respect to earth, and opened relay RLY 2. No further pulses would then pass to V.13. When the pre-set interval was finished and V.15 returned to its quiescent state a pulse from anode 1 of V.15 was passed through C.6 to the grid 1 of V.10 and momentarily energised relay RLY 1. This allowed all the electrodes except the zero electrode of dekatrons V.1, 2, 5 and 6 to rise above earth and re-set them ready for the next cycle of operation. At the same time, relay RLY 2 closed and the next initiating pulse for the trigger circuit V.13 was allowed to pass to grid 1 of this valve. The whole cycle then repeated itself. Switches S.1 and S.2 when in the open position, stopped the circuit from operation. Switch S.2 when operated to close C.6 to the high tension supply would re-set all counters. Switch S.1 when closed in the position where the paired contacts were made would re-set the dekatrons and initiate one single cycle of operation, and is used to pre-set the number of pulses

and the timing interval before starting to use the instrument. For normal operation, both S.1 and S.2 were closed and in the position where C.6 was connected to anode 1 of V.15, and the out-put of the multi-vibrator was connected to grid 1 of V.13.

The power supply for this circuit was designed according to BENSON (1949), the negative supply being obtained by full wave rectification, using a metal rectifier and a stabilivolt valve STV 280/40; The smoothing for this negative supply was carried out in the conventional manner. The 500 v supply was used unstabilised. Any conventional power supply capable of giving a positive potential of 300 to 500 v and a negative potential of 280–300 v, could be used to drive the instrument.

Electrode vessels

For manometric work, most use has been made of conical electrode vessels *A* (McILWAIN, 1951*a*) and *E* (McILWAIN, 1951*b*) and these are supplied by Messrs. A. W. Dixon & Co., 30 Anerley Station Road, London, S.E.20. The grid electrodes *H* (AYRES and McILWAIN, 1953) employed with vessels *A*, are supplied by Mr. H. L. Buddle, 46 Cedarcroft Road, Chessington, Surrey.

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Vessels *A* and *E* as received from the glassblower are prepared for use as follows. (i) They are tested for possible leaks, especially at the electrode seals, by filling the vessels with water, connecting to a gas cylinder with a pressure gauge, and taking to 10 lb/in^2 for 30 min. Any with leaks are rejected. (ii) Their volume is measured with mercury by conventional methods. (iii) Permanent external connections are made at the electrode side-arms, which contain a stub of tungsten wire. One of these is just covered with solid sodium nitrite; the vessels and side arm must be dry before this is done. The side arm is then heated in the region of the stub with a small luminous Bunsen flame until the nitrite gently bubbles. As much as possible of the nitrite is then poured off, the vessel allowed to cool, and the remaining nitrite washed out with water. The tungsten should have changed from its previous black colour to a clean matt metallic grey. If necessary the process is repeated; caution is needed in heating as the process can become violent. The second side arm is treated similarly, and then both are filled with a copper-plating solution (2 g of copper acetate monohydrate; 2·2 g of KCN; 2 g of $Na_2S_2O_5$; 2·5 g/100 ml of $Na_2CO_3.10H_2O$). Inside the vessel, the platinum end of a stub is connected to the cathode of a 3 v battery whose anode goes to a thin copper wire which is inserted in the plating solution in the corresponding side arm. Current is passed for 10 min at about 15°. The second side arm is similarly treated, both washed out with water, zinc chloride flux added and most of this shaken out, leaving a little adhering to the stubs. Solder is melted in the side arms by careful heating and the excess tipped out, leaving the tungsten tinned.

Lengths of tinned copper wire (16 S.W.G.; 1·62 mm diameter) are prepared to fit the side arms and to project about 15 mm beyond them, and blobs of solder placed on their lower ends. One wire is held in a side arm with its solder against the tinned tungsten and the two joined by gently heating from a Bunsen flame. Rings of pasteboard are cut with corkborers so that they fit the annular space between the copper wire and the glass of the side arm, and one is pushed about 6 mm down each side arm. The space above it is filled with glycerol-litharge cement. The completed electrode arm resembles that of Fig. 2*E* of AYRES and McILWAIN (1953) but has less solder. (iv) The portions of platinum wire within the electrode vessels, being either electrodes or stubs to carry electrodes, are gold plated as described by McILWAIN (1951*a*).

Electrodes for localized application of pulses

The electrodes described earlier in this and in previous papers, were designed in order to give maximum metabolic response by the tissue to a given stimulus. In electrode vessel *E* (McIlwain, 1951*b*) tissue floats freely between the electrodes; in electrodes *H* (Ayres and McIlwain, 1953) the tissue is within wires whose bared

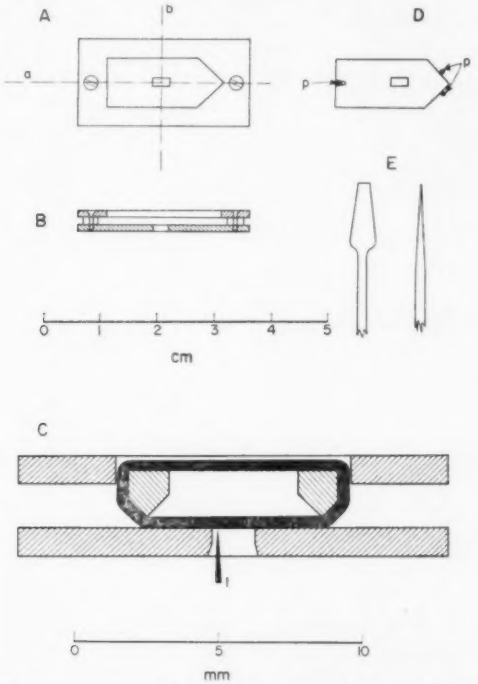

Fig. 3.—Templates for preparing electrodes for localized application of electrical pulses. *A–C:* trough to take the upper electrode while a length of 1·5 mm of one enamelled wire is exposed. *A:* plan; *B:* section at the midline *a*; *C*, enlarged section at *b* of the template in use, with the upper electrode in position and being scraped by the tool *t*. Two views of the scraping head of the tool are shown in *E*. *D:* template for similarly exposing a 1·5 mm length of a wire of the lower electrode; *p*, points at which the clips of the lower electrode grip the template.

surfaces form a grid at about 2 mm intervals. Recently, electrode arrangements have been sought which might permit observation of transmission in the tissue of effects from a localized application of pulses. An arrangement found successful from this point of view is based on the grid electrodes *H*, with however their enamelled wires bared at small areas only (Bollard and McIlwain, 1959; Kurokawa, 1960; McIlwain, 1960). It has been termed electrode *L* (localized) and is prepared as follows.

Electrodes *H* (Ayres and McIlwain, 1953, Fig. 2*A–D*) are received with wire completely enamelled. Their upper portion is placed in a trough of a template (*A–C*, Fig. 3, this paper) shaped to take accurately and firmly all electrodes of the set being used. The template has in its base a slit which exposes a limited length of one of the electrode wires. The length of wire exposed is governed by the width of the slit, which has been made 1·5 mm at the point at which it meets the wire.

This length of wire is now scraped bare of insulation, through the slit, with a tool *t* made from a lancet. The lower part of the electrode is similarly treated after a second template (*D*, Fig. 3) has been clipped to it. This preparation enables experiments to be run with slices held in position by the grid of enamelled wires and exposed to pulses from two electrodes 1·5 mm in length and 2 mm apart, at defined points of the slice; metabolic results have been reported by BOLLARD and McILWAIN (1959), KUROKAWA (1960) and by McILWAIN (1960).

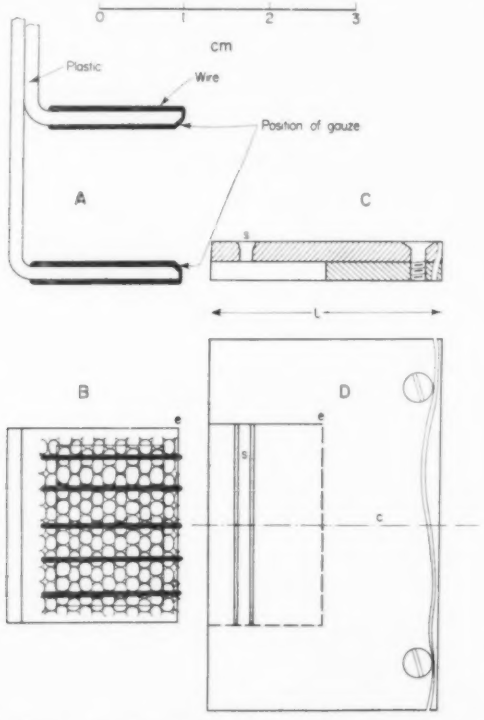

FIG. 4.—*A*, *B*: jaws of quick-transfer holder with nylon gauze, drawn in their open position. *A*: end elevation, showing the points at which the nylon gauze is inserted. *B*: plan of the lower jaw of the holder with the gauze insert. *C*, sectional elevation at *c* and *D*, plan of part of the template for localized exposure of wires of the holder. Its total length *l* is 5 cm; *s*, slot 1·5 mm in width below; *e*, *e*: edges of template and quick-transfer holder which are brought together in using the template.

Quick-transfer apparatus

The quick-transfer apparatus previously described (HEALD and McILWAIN, 1956) holds a relatively large tissue slice at points near its edge, in such a way that the slice can be rapidly transferred from an incubating to a fixing solution. In one version of the apparatus electrical pulses can be applied to the slice. The scope of the apparatus has now been extended by fixing fine nylon nets to its jaws (Fig. 4) so that slices of many sizes, and also tissue fragments, can be treated in the same way. During incubation the fragments are held between the nets. This does not interfere with access of materials to the slice, for KUROKAWA (1960) found the phosphocreatine of slices of cerebral tissues to be adequately maintained in the apparatus, and this

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constituent of the tissue has been found to be sensitive to inadequacies in the supply of oxygen and glucose (HEALD, MCILWAIN and NELLIGAN, 1954). The nylon net found suitable was kindly characterized by British Nylon Spinners Ltd. as follows: wt/yd², 0·42 oz; yarn denier, 45 (15)/Z; resultant denier, 58; mesh size 1·84 × 1·39 mm.

Quick-transfer holders with net and locally-bared electrodes have been used in observing change in tissue composition in response to local application of electrical pulses (KUROKAWA, 1960). In this study, slices of subcortical white matter of the guinea pig were employed and these are limited to about 1 cm² in area. The nylon net was necessary in order to hold the slice without folding and in a definite position in relation to two 1·5 mm lengths of exposed silver wire. The localized exposure of the enamelled electrode wires was carried out by methods similar to those described above, using the template illustrated in Fig. 4 *C* and *D*. This is made from two sheets of hard transparent plastic (Perspex) 4 × 5 × 0·18 cm, cut away as shown in Fig. 4 and held firmly together by the two screws which are shown. The same template serves for the upper and for the lower jaw of the quick-transfer holder. In using it on the lower jaw, the template as shown in *D* is brought over the jaw *B* by moving it to the left until edge *e* of the lower sheet of the template, meets the edge *e* of the jaw of the holder, and the enamelled wire of the holder rests against the 1·5 mm slot of the template. Enamel is then removed with the tool of Fig. 3.

DISCUSSION

The apparatus described or referred to above, enables several types of variable to be studied in experiments in which the metabolism of isolated neural tissues is stimulated by electrical pulses. (i) The voltage–time relationships of the pulses can be varied from exponential to sine-wave currents. It is important to note that the pulses remain of alternating polarity. Polarization of metal surfaces in aqueous solutions is such that after the first or the first few pulses of a given polarity, resistance at the metal–fluid interface greatly increases; it may however be brought to its original value by a pulse of the opposite polarity. (ii) The pulses can be varied in duration, in potential and in frequency. This allows the excitability of tissue samples to be characterized by determination of threshold potentials and of potential–response and duration–response curves. (iii) The series of pulses can be interrupted, so displaying any after-effects. (iv) The pulses can be applied locally, so displaying any spread of their effect through a tissue sample. (v) A variety of metabolic sequelae to pulses can be examined. These relate not only to respiration and carbohydrate metabolism, for in addition the quick-transfer electrodes enable tissue to be fixed rapidly for analysis of tissue constituents.

SUMMARY

A simplified apparatus is described for generating a series of condenser-pulses for applying to neural tissues immersed in aqueous fluids during metabolic experiments. A pulse-interruptor and timing circuit is described which yields from such series chosen groups of pulses at chosen intervals.

Electrodes for localized application of pulses to tissues, and for the rapid transference of tissue samples for extraction and analysis, are also detailed.

Acknowledgements—I am greatly indebted to Mr. A. McNeil and Mr. J. G. Platt, and to those mentioned above, for their part in the construction and design of the apparatus.

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PRELIMINARY NOTES

Formation of 3:4:5-trimethoxyphenylethanol from mescaline*

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THE metabolism of mescaline (3:4:5-trimethoxyphenylethylamine) is not yet well understood. Since the behavioural effects in animals occur after mescaline has apparently disappeared from the brain, it is believed that a metabolite of this compound may be the active agent (BLOCH *et al.*, 1952). Also the previous finding (GOLDSTEIN *et al.*, 1960) that primary alcohols are metabolites of dopamine suggested that mescaline may undergo similar metabolic changes. Therefore, the formation of 3:4:5-trimethoxyphenylethanol from mescaline was investigated in groups of rats which were treated as follows: Group 1 with 400 μg/kg mescaline-1-^{14}C; Group 2 with 400 μg/kg mescaline-1-^{14}C and 100 mg/kg iproniazid; Group 3 with 400 μg/kg mescaline-1-^{14}C and 2 ml of a 6·3% suspension of disulphiram in 25% aqueous ethanol; and Group 4 with 400 μg/kg mescaline-1-^{14}C and 50 mg/kg per os calcium carbimide.

Twenty-four hour urines were collected from each group and hydrolysed with glusulase. The urines were adjusted to pH 2 and treated four times with ethyl acetate. The ethyl acetate extracts were concentrated, a portion was chromatographed in chloroform–acetic acid–water (2:1:1), and the fractions, separated were subjected to chromatography in *iso*propyl alcohol–aqueous ammonia–water (8:1:1). One radioactive peak, detected on each of the *iso*propyl alcohol–aqueous ammonia chromatograms, had the same mobility as 3:4:5-trimethoxyphenylacetic acid. However, the material corresponding to this peak was not homogeneous. The material in this peak was a mixture of 3,4,5-trimethoxyphenylacetic acid and 3,4,5-trimethoxyphenylethanol, since the mobilities of both were found to be the same in this solvent system. In order to separate these two compounds the radioactive material was eluted with methanol and then acetylated (GOLDSTEIN, 1959). The acetylated alcohol is extractable into methylene chloride, while the acid remains in the aqueous bicarbonate layer. The acetylated radioactive material was chromatographed in the Bush 'C' solvent system and had the same mobility as acetylated authentic 3:4:5-trimethoxyphenylethanol.

In untreated rats (group 1) very little 3:4:5-trimethoxyphenylethanol was formed (Table 1), while in rats treated with iproniazid (group 2) a decrease in the amount of deamination occurred although the alcohol formation was enhanced. Since disulphiram enhanced the formation of alcohol (SMITH *et al.*, 1960), and treatment with ethanol also enhanced alcohol formation from amines, we investigated the combined effects of disulphiram and ethanol upon the formation of alcohol from mescaline. 3:4:5-Trimethoxyphenylethanol formation was indeed higher (Table 1) in disulphiram–ethanol treated rats (group 3) than in rats treated with either compound alone. Another aldehyde oxidase inhibitor, calcium carbimide, also enhanced alcohol formation markedly (group 4).

These experiments suggest that a pathway for the transformation of mescaline involves its oxidation to 3:4:5-trimethoxyphenylacetaldehyde, and then follows further oxidation to 3.4:5-trimethoxyphenylacetic acid or reduction to 3:4:5-trimethoxyphenylethanol. To our knowledge it has not previously been demonstrated that iproniazid inhibits deamination of mescaline *in vivo*. This is shown by the increase in excretion of unmetabolized mescaline and the decrease in excretion of acid in iproniazid-treated rats.

* This work was supported by grants from the National Institutes of Health and the Parkinson's Disease Foundation.

It is also of interest to note that 3:4:5-trimethoxyphenylethanol formation from mescaline and 3-methoxy-4-hydroxyphenylethanol formation from dopamine are both influenced to the same extent by the above mentioned compounds (GOLDSTEIN, 1960).

TABLE 1.—THE RADIOACTIVITY OF MESCALINE AND ITS METABOLITES ISOLATED FROM RATS' URINE

	Counts/min in 24 hours urine		
Group	Mescaline	3:4:5-Trimethoxy-phenylacetic acid	3:4:5-Trimethoxy-phenylethanol
1	15,000	75,000	7,500
2	41,500	32,000	11,500
3	29,000	41,000	20,000
4	26,000	35,000	30,000

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SUMMARY

3:4:5-Trimethoxyphenylethanol was isolated from the urine of rats pre-treated with mescaline-1-^{14}C. The excretion of this alcohol was increased by pre-treatment of the rats with iproniazid which also inhibited the conversion of mescaline to 3:4:5-trimethoxyphenylacetic acid. Pre-treatment with a disulphiram–ethanol mixture or calcium carbimide also markedly enhanced the conversion of mescaline to 3:4:5-trimethoxyphenylethanol. These experiments demonstrate that a pathway for the transformation of mescaline involves its oxidation to 3:4:5-trimethoxyphenylacetaldehyde, and then follows further oxidation to 3:4:5-trimethoxyphenylacetic acid or reduction to 3:4:5-trimethoxyphenylethanol.

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Normal excretion of homovanillic acid in the urine of patients with Huntington's chorea

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HUNTINGTON's chorea, a disease which is frequently inherited in single autosomal dominant fashion, is associated with wide-spread neuronal degeneration of the brain; however, the caudate and the putamen are the nuclear masses most severely and consistently involved. The high concentrations of dopamine (3:4 dihydroxyphenylethylamine) found in the caudate nucleus (5·9 μg/g) of the dog by CARLSSON (1949) and in the caudate nucleus (5·7 μg/g) and in the putamen (8·3 μg/g) of the human by SANO *et al.* (1959) in comparison with other parts of the brain, such as the cerebral cortex (0·07 μg/g)

have led to the supposition that the function of dopamine in these areas may be more than simply that of a precursor of norepinephrine since the latter is not similarly concentrated in these areas. Because of the genetic component of Huntington's chorea, therefore, it seemed logical to look for evidence of a biochemical abnormality in the metabolism of dopamine in patients with this disease. This was begun by measuring the urinary excretion of 3-methoxy-4-hydroxyphenylacetic acid (homovanillic acid, HVA) which is the major urinary metabolite of exogenous (and presumably endogenous) dopamine.

Four ml aliquots of 24-hour urine specimens acidified to pH 1·5 (conc. HCl) were analysed by a modification of the method of SHAW *et al.* (1957): after saturation with NaCl the urine was extracted 3 times with half volumes of ethyl acetate; the combined ethyl acetate extracts were reduced to a small volume under a stream of air at 37° and examined by 2-dimensional chromatography in *iso*propyl alcohol–ammonia–water (8:1:1, ascending) followed by benzene–propionic acid–water (2:2:1, organic phase, descending). After drying, the paper chromatograms were sprayed with a freshly prepared mixture of diazotized sulphanilic acid (25 ml of 0·3% sulphanilic acid in 0·8 N-HCl and 1·5 ml of 5% $NaNO_2$) and 10% Na_2CO_3 (1:3). Standards (25 and 50 μg) of authentic HVA (California Foundation for Biochemical Research) were carried through the extraction procedure and 50 μg standards were added to duplicate portions of urine to correct for losses in extraction of HVA from urine. When dried, the HVA spots were cut out and eluted for 15 minutes in 5 ml of 0·1 M-phosphate buffer pH 7·0 and then read in a Beckman DU spectrophotometer at 508 mμ. Recoveries of added HVA averaged 94 per cent and results are corrected for these losses. The accuracy of the method is $\pm$10 per cent. The urine collections of the first three patients were made from indwelling catheters and are believed to be complete. Creatinine was measured by the Jaffe reaction. The mean excretion of HVA, calculated either as total mg HVA or μg/mg creatinine of

TABLE 1.—URINARY HOMOVANILLIC ACID EXCRETION

Normal controls			Huntington's chorea		
Patient (sex	Homovanillic acid (mg/24 hr)	Homovanillic acid (μg/mg) creatinine	Patient (sex)	Homovanillic acid (mg/24 hr)	Homovanillic acid (μg/mg) creatinine
S.M. (F)	5·9, 2·4	4·5, 2·0	M.K. (F)	3·6, 3·1	5·7, 3·6
R.K. (M)	12·1, 17·1	5·2, 6·8	D.C. (M)	8·2, 5·9, 6·1	2·3, 5·8, 4·3
M.M. (M)	11·0	8·6	I.J. (M)	4·9, 6·6, 1·9	14·2, 6·9, 6·4
R.Y. (F)	6·9	5·5	R.C. (M)	8·0	6·9
S.Y. (M)	11·8	6·1			
Mean (SD)	9·6 (1·9)	5·5 (0·8)	Mean (SD)	5·4 (0·7)	6·2 (1·1)

Patient M.K. was on Sparine at the time of the urine collection.
Patients D.C. and M.K. had severe chorea and dementia but no family history of a similar disease and were therefore presumably cases of sporadic Huntington's chorea.
Each value is the mean of duplicate determinations.

the patients with Huntington's chorea was not significantly different from the corresponding values for the normal controls. The upper limit of our range of HVA for normal controls is somewhat higher than the range of 3 to 8 reported by SHAW *et al.* (1957).

BARBEAU (1960) has reported the presence of large amounts of a catecholamine-like substance in the urine of 5 patients with Huntington's chorea and discussed the possibility that it might be dopamine. Since dopamine is ordinarily metabolized almost entirely to homovanillic acid (GOLDSTEIN *et al.* (1959)), our observations that urinary HVA excretion is normal in patients with Huntington's chorea makes this particular possibility unlikely.

Four patients with Huntington's chorea did not differ from normal controls in the urinary excretion of homovanillic acid, a major terminal metabolite of dopamine. It is inferred that the metabolism of dopamine is not altered in this disease.

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Papers to be published in future issues

J. L. CHASON and A. G. E. PEARSE: Phenazine methosulphate and nicotinamide in the histochemical demonstration of dehydrogenases in rat brain.

L. ERNSTER and L. HERLIN: The fate of intra-cisternally injected tracer phosphate in the rabbit, normally and as influenced by sulphhydryl reagents. A kinetic approach to the mechanism of the blood-brain barrier.

N. A. TUQAN and C. W. M. ADAMS: Histochemistry of Myelin—I. Proteins and lipid-protein complexes in the normal sheath.

C. W. M. ADAMS and N. A. TUQAN: Histochemistry of Myelin—II. Proteins, lipid-protein dissociation and proteinase activity in Wallerian degeneration.

B. F. S. Bailey and P. J. Heald: The separation of the cytoplasmic proteins of brain by electrophoresis in a starch-gel medium.

M. H. APRISON and C. B. FERSTER: Neurochemical correlates of behaviour—II.

G. J. ALEXANDER: Choline biogenesis—I. Utilization of aminoethanol-1:2-C^{14} in cell-free homogenates.

G. J. ALEXANDER: Choline biogenesis—II. The effect of homocysteine on serine metabolism.

G. J. ALEXANDER: Choline biogenesis—III. The origin of the three methyl groups.

L. L. UZMAN, M. K. RUMLEY and S. VAN DER NOORT: The substrate specificity of mouse brain peptidase activity.

R. KUTTNER, J. A. SIMS and M. W. GORDON: The uptake of a metabolically inert amino acid by brain and other organs.

W. C. CURTIS and J. H. SEIPEL: Determination of total-lipide in the cerebrospinal fluid by paper chromatography.

K. D. NEAME: Uptake of amino acids by mouse brain slices.

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PHENAZINE METHOSULPHATE AND NICOTINAMIDE IN THE HISTOCHEMICAL DEMONSTRATION OF DEHYDROGENASES IN RAT BRAIN

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HISTOCHEMICAL methods for oxidative enzymes have been applied to the central nervous system with varying results. Until recently only cytochrome oxidase (ROIZIN, 1955) and succinate dehydrogenase (NACHLAS *et al.*, 1957) had been demonstrated in a satisfactory manner. Acceptable methods for the so-called DPN diaphorase† have lately become available. Although the histochemical localization of some DPN-linked respiratory enzymes has been described in the literature, the methods used were undoubtedly inadequate. The purpose of this study, therefore, has been to develop methods for such pyridine nucleotide-linked enzymes and to increase, if possible, the sensitivity of existing methods for oxidative enzymes needing no co-enzymes. Such methods should be particularly useful in the study of diseased neurones, permitting the earliest metabolic changes to be localized and recorded.

As an auxiliary part of the work comparisons have been made between the observed histochemical results and those derived from microchemical and macrochemical assays.

MATERIALS AND METHODS

Various breeds of healthy rats of both sexes, weighing 175–200 g and on a standard laboratory diet were used. The animals were sacrificed under light ether anaesthesia by transection of the lower cervical spinal cord. A 4 mm complete coronal section of the cerebrum at the level of the infundibulum, a 4 mm complete transverse section of the cerebellum and pons, a 6 mm longitudinal section of the cervical spinal cord and a 4 mm section of liver were removed, chilled (quenched) to $-70°$ with dry ice, and attached to tissue holders. The frozen tissues were maintained at $-20°$ until cut, usually within 4 hr, always less than 24 hr. The tissues were cut at 6 and 18 μ in the cold microtome (cryostat), mounted directly onto coverslips and maintained in the moist state in the microtome cabinet ($-20°$) until cutting was completed (15–30 min). The sections were removed, covered immediately with 0·1–0·2 ml of incubating medium and incubated in air at 37° for 15–30 min. Reactions were stopped and the tissues simultaneously fixed at the end of this period by immersion for 10 min in 15% formol–calcium. The sections were then mounted in plain glycerine jelly. After 24 hr the coverslips were ringed with nail polish to minimize drying and crystallization of the formazan deposits. To aid in the identification of cells and areas counterstaining was sometimes carried out, using either haematoxylin and eosin or Solochrome cyanine R.

Incubating Media

Succinate dehydrogenase. The medium described by PEARSE (1960) was prepared by mixing 5·0 ml of an 0·66 M-phosphate buffer (pH 7·4) with 0·6 ml of an 0·5 M-cobaltous chloride solution.

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† The following abbreviations are used throughout this paper. DPN, diphosphopyridine nucleotide; DPNH, reduced diphosphopyridine nucleotide; INT, 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride; MTT, 3-(4:5-dimethylthiazolyl-2)-2:5-diphenyl tetrazolium bromide; Nitro-BT, 2:2′ di-*p*-nitrophenyl-5:5′-diphenyl-3:3′-(3:3′-dimethoxy-4:4′-biphenylene) ditetrazolium chloride; PMS, phenazine methosulphate; PVP, polyvinyl pyrrolidone; TPN, triphosphopyridine nucleotide; Tris, Tris (hydroxymethyl) aminomethane.

The mixture was filtered. To 2·5 ml of filtrate were added 2·5 ml of MTT solution (1 mg/ml) and 3·0 ml of sodium succinate (0·57 g/10 ml). The pH was adjusted to 7·0 with stock Tris buffer (pH 10·4) using pH test paper. Following this the solution was made up to 10 ml with distilled water and 0·375 g of PVP were dissolved in the medium (this is half the amount recommended for other tissues).

DPN diaphorase. The medium described by SCARPELLI, HESS and PEARSE (PEARSE, 1960) but with one-tenth the quantity of PVP was used. The stock solution was made by mixing 5·0 ml of an 0·06 M-phosphate buffer (pH 8·2) and 0·6 ml of 0·5 M-cobaltous chloride and filtering. To 2·5 ml of filtrate were added 2·5 ml of MTT solution (1 mg/ml) and distilled water to make 8 ml. The pH of this mixture was adjusted to 7·2 with stock Tris buffer (pH 7·4) using the glass electrode. The solution was made up to 10 ml with distilled water and 0·075 g PVP added. The stock solution kept frozen at −20° retains its activity unaltered for at least 6 weeks.

The incubating medium was made by adding 6 mg of DPNH to 1 ml of stock solution warmed to room temperature immediately before use. A final adjustment of the pH to 7·0 with Tris buffer (pH 8·5), using pH test paper, is made at this time.

DPN-linked dehydrogenases. The DPN-linked oxidative enzymes studied included malate, lactate and α-glycerophosphate dehydrogenases. The incubating media were made from a stock solution identical with that of the DPN-diaphorase stock, to 10 ml of which were added 0·732 g of nicotinamide. This stock solution maintains its activity unaltered for at least 6 weeks when kept frozen at −20°.

The substrates used were all molar solutions. The malate solution was made using sodium L-malate adjusted to pH 7·0 with 0·1 N-NaOH. The lactate substrate was made by adding 8·4 ml distilled water to 1·6 ml of a commercially available 70% solution of sodium DL-lactate at pH 7·0. The α-glycerophosphate solution was made with sodium DL-α-glycerophosphate. Its pH was adjusted to 7·0 with 0·1 N-HCl.

Each incubating medium, made just prior to use, consisted of 0·9 ml of stock solution, 0·1 ml of M-substrate and 6 mg DPN. The final adjustment of pH to 7·0, made immediately after mixing, was done with Tris buffer (pH 8·5) and pH test paper. Six mg PMS were sometimes also added to the incubating medium.

In all of the methods Nitro-BT and sodium cyanide in the proper amounts (PEARSE, 1960) can be substituted for MTT and cobaltous chloride, with good results. In our opinion the MTT and cobaltous chloride method is more sensitive. The granules are smaller and the red background staining seen with Nitro-BT and sodium cyanide does not occur.

Controls. Sections of liver, run in parallel with those of the central nervous system, were used as positive controls. Media controls consisted of omissions of DPN, DPNH, substrate and nicotinamide, separately and in combination. In all instances in the appropriate methods these resulted in a negative reaction in the central nervous system and liver (here with the exception of nicotinamide).

RESULTS

Succinate dehydrogenase

With succinate dehydrogenase, the strongest reaction was in the ependymal cells of the choroid plexuses, cervical central canal and ventricles (Fig. 1). The ependymal cells lining the hypothalamus, and in the region of the infundibulum, were exceptional in reacting only weakly. The intensity of the reaction in the Purkinje cells (Figs. 2 and 3) was generally equal to that of ependyma. The large cells of the pontine reticular substance and nuclei, many cells in the temporal allocortex (Fig. 4), and some in the thalamus, hypothalamus and caudate nucleus reacted equally strongly. Slightly less intensely reacting were the nerve cells of the frontal and parietal cortices, the remainder of the cells in the pons, and in the thalamus (Fig. 1), hypothalamus, and adjoining nuclei of the internal granular and molecular layers of the cerebellum (Figs. 2 and 3). There was no apparent laminar concentration of formazan granules in the cerebral isocortex with the exception of the less intensely stained first layer. In the cerebellum the Purkinje layer was most deeply stained, the internal granular layer slightly less, and the molecular layer slightly less still (Figs. 2 and 3). In the 18 μ sections gradually

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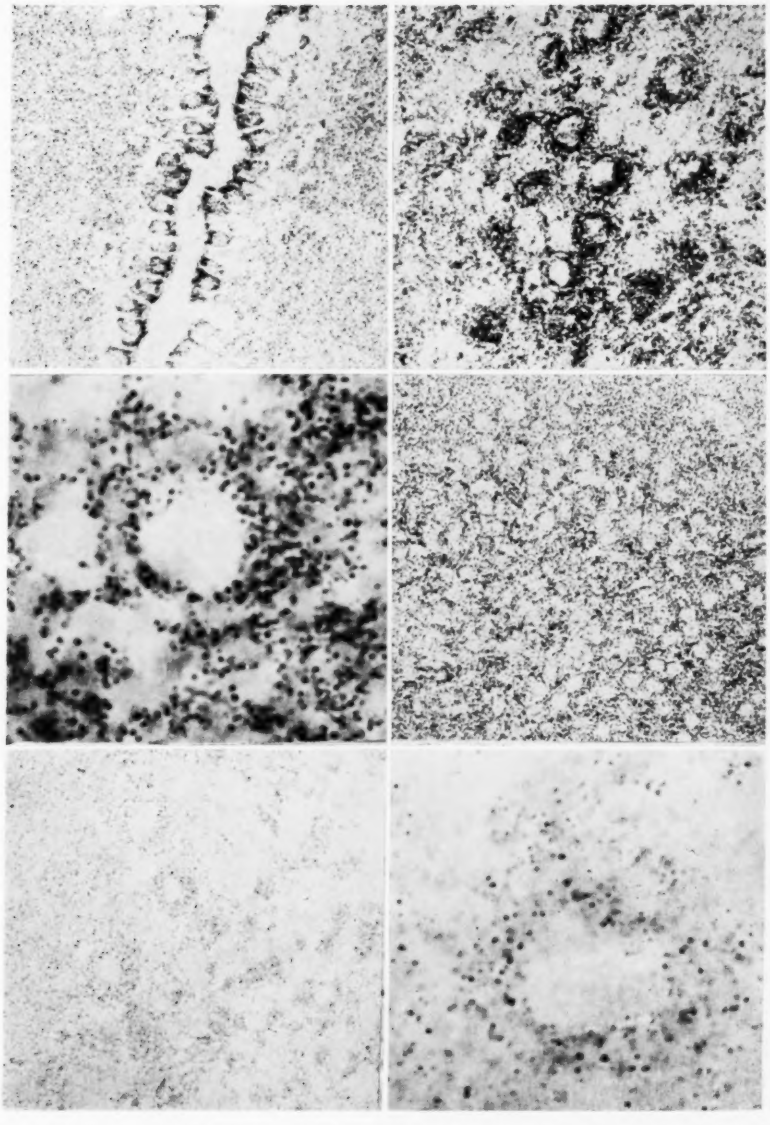

FIG. 1 (Top left).—Ependyma and adjoining thalami. Succinate dehydrogenase using MTT. 6 μ $\times$347.

FIG. 2 (Top right).—Cerebellum. Molecular layer on left with Purkinje cells and internal granular layer toward the right. Succinate dehydrogenase using MTT. 6 μ $\times$347.

FIG. 3 (Middle left).—Purkinje cell with non-staining nucleus and nucleolus surrounded by internal granular cells. Same as Fig. 2. 6 μ $\times$1800.

FIG. 4. (Middle right).—Temporal lobe allocortex. Succinate dehydrogenase using MTT. 6 μ $\times$347

FIG. 5 (Bottom left).—Cerebellum. Molecular layer on left with Purkinje and internal granular cells toward right. DPN diaphorase using MTT. 6 μ $\times$347.

FIG. 6 (Bottom right).—Purkinje cell with non-staining nucleus and nucleolus and internal granular cells. Same as Fig. 5. 6 μ $\times$1800.

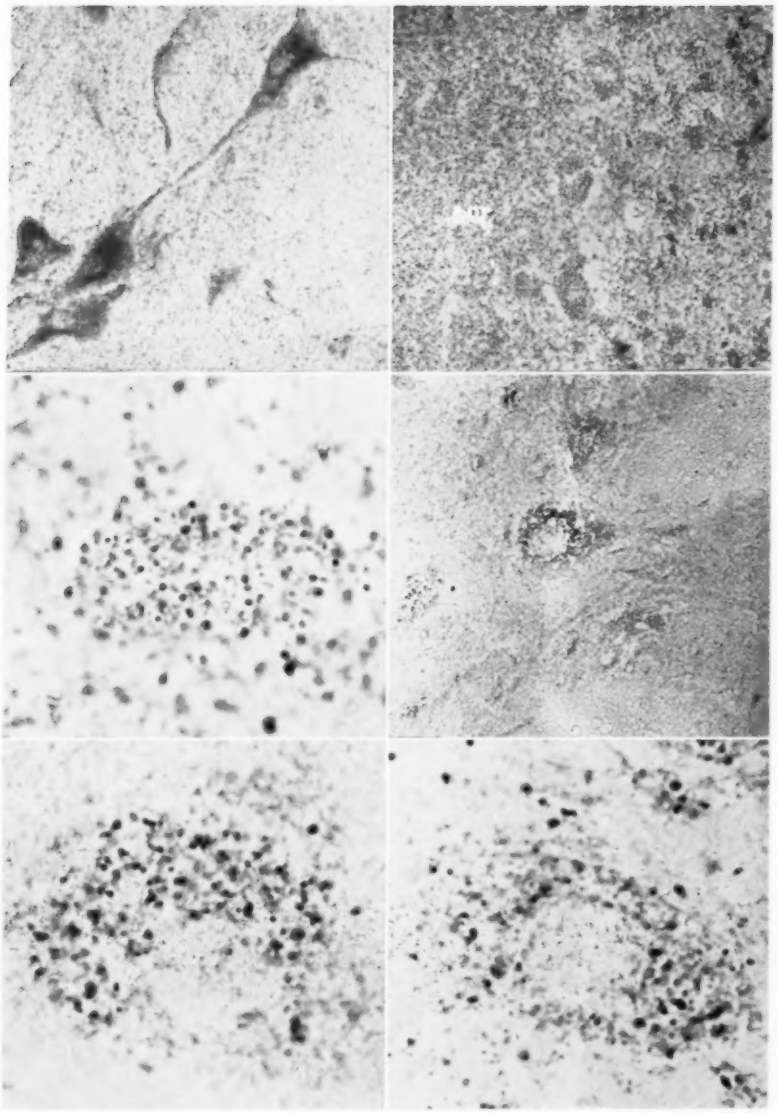

FIG. 7 (Top left).—Pons. Cell of reticular substance demonstrating granules in cell processes. DPN diaphorase using MTT. 18 μ ×369.

FIG. 8 (Top right).—Cerebellum. Molecular layer on left. Purkinje and internal granular cells toward right. Malate dehydrogenase using MTT and PMS. 6 μ ×347.

FIG. 9 (Middle left).—Purkinje cell and internal granular cells. Same as Fig. 8. 6 μ ×1800.

FIG. 10 (Middle right).—Pons with cells of reticular substance. Note non-staining nucleus. Lactate dehydrogenase using MTT. 6 μ ×347.

FIG. 11 (Bottom left).—Purkinje cell with non-staining nucleus. Lactate dehydrogenase using MTT. 6 μ ×1800.

FIG. 12 (Bottom right).—Purkinje cell with non-staining nucleus. α-Glycerophosphate dehydrogenase using MTT and PMS. 6 μ ×1800.

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decreasing numbers of formazan granules could be traced from the perikaryon into both axones and dendrites.

The relatively few granules in the white matter could usually be attributed to a nerve cell or its processes extending from the adjoining cortex, to microglia, or to the cells forming blood vessels. A few small clusters of granules surrounded nuclei resembling those of astrocytes and oligodendrocytes.

The uniform reaction of the cells of the pia-arachnoid was equal in intensity to that of the more deeply stained ependymal cells. Cells in all layers of the blood vessels were strongly reactive. The degree of staining of the muscle cells of the larger arteries was similar to that of the Purkinje cells. With decrease in arterial size there was an accompanying decrease in reactivity. A few formazan granules were regularly present in capillary endothelium.

DPN diaphorase

The DPN diaphorase reaction, like that for succinate dehydrogenase, was strongest in the pia-arachnoid and large arteries and slightly less strong in the ependymal cells lining the choroid plexuses, cervical central canal and ventricles. As with succinate dehydrogenase a weak response was given by the cells lining the hypothalamus and infundibulum. The smaller arteries, arterioles and capillaries, although reacting less strongly than the above mentioned components, were easily located in the weakly stained cortex and almost unstained white matter. The granularity of the nerve cells, although slightly less than that seen with succinate dehydrogenase, followed the same pattern (Figs. 5, 6 and 7). The occurrence of a few scattered granules in astrocytes and oligodendrocytes could not be confirmed since specific counterstains could not be used.

DPN-linked dehydrogenases

No reaction was obtained with malate, lactate or α-glycerophosphate dehydrogenases until a sufficient quantity of nicotinamide had been added to the medium. A very high final concentration (0·57 M) was necessary to achieve the required degree of inhibition of the strong diphosphopyridine nucleotidase present in rat brain.

The observed reaction rates for the three enzymes, when the full amount of nicotinamide was present in the incubating medium, were in all cases lower than for DPN diaphorase. With all three the pattern of formazan deposition was similar to that of the diaphorase. The malate (Figs. 8 and 9) and lactate (Figs. 10 and 11) dehydrogenases were much more active than α-glycerophosphate dehydrogenase (Fig. 12). Satisfactory demonstration of the latter could only be made with the thicker (18 μ) sections. Although the addition of PMS appeared initially to raise the reaction rate for all three enzymes, no increase was observed after incubation for 15 and 30 minutes.

DISCUSSION

Of the five enzymes studied in the central nervous system two are concerned with glycolysis (α-glycerophosphate and lactate dehydrogenase) and two form part of the citric acid cycle (succinate and malate dehydrogenase). A recent suggestion has been made, however, that α-glycerophosphate dehydrogenase may be functionally related to the succinate dehydrogenase system (RINGLER and SINGER, 1958). The so-called DPN diaphorase is a lipoflavoprotein enzyme capable of transferring electrons from

DPNH to certain dyes (MEHLER, 1957). The histochemical reaction demonstrates the sites and relative amounts of the lipoflavoprotein present in the mitochondria of individual cells. The result may represent the total potential activity of each of the DPN-linked systems (PEARSE, 1960), but its true significance is not known.

Succinate dehydrogenase

Increases in sensitivity and precision of an histochemical method for succinate dehydrogenase have made possible its accurate localization and estimations of its relative quantity. The enzyme was found by LEDUC and WISLOCKI (1952) to be more concentrated in the choroidal epithelium of the rat with lesser amounts in the cells of the intercolumnar tubercle, pineal gland and neurohypophysis. In the same year PADYKULA (1952) reported that in rat brain greater amounts of enzyme were present in the grey matter, especially in the Purkinje cells, than in the white matter. RUTENBERG, WOLMAN and SELIGMAN (1953), using six different mammals, found that the enzyme was restricted to grey matter, particularly the perikaryon of nerve cells and some cell processes. They could not demonstrate activity in glial cells. Similar localizations were demonstrated in biopsy specimens of human cerebrum and cerebellum by MUSTAKALLIO (1954).

More recently, the localization of succinate dehydrogenase in the cells of the human and animal central nervous system has been extended to include most, if not all, of the nerve cells and some of their processes. The neuropil, the cells of the leptomeninges, and those of blood vessels larger than capillaries have been included in the list. Positive identification of succinate dehydrogenase has been made in astrocytes and oligodendrocytes, although the means by which these cells were distinguished is nowhere given (NACHLAS *et al*, 1957; SHIMIZU and MORIKAWA, 1957; SHIMIZU, MORIKAWA and ISHI, 1957; FRIEDE, 1958, 1959*a*, *b*; KIVALO, RINNE and MÄKELÄ, 1958; ODA *et al*., 1959; POTANOS, WOLF and COWEN, 1959; WOLFGRAM and ROSE, 1959).

With the MTT method all of these localizations are shown as well as extension of the enzyme for some distances into the majority of nerve cell processes, and its presence in endothelial cells and in microglia. The significance of the negative or weak reaction of the ependymal cells in the region of the hypothalamus and infundibulum as well as in the cells of the intercolumnar tubercle, pineal and neurohypophysis (LEDUC and WISLOCKI, 1952; SHIMIZU and MORIKAWA, 1957; SHIMIZU, MORIKAWA and ISHI, 1957; FRIEDE 1959*a*) remains unexplained.

Difficulties in cutting, the production of damaging tissue artifacts, and the loss of succinate dehydrogenase activity with temperatures below $-1°$ to $-3°$ have been reported by FRIEDE (1958), who used 60 μ sections for most of his work. These problems did not occur with the freezing and cutting techniques employed in this investigation. Using MTT, the reactions were too intense to be evaluated accurately in sections more than 25 μ thick. In our work the sole value of the thicker (18 μ) sections lay in the demonstration of granules in cell processes which maintained their attachment to the body of the cell.

DPN diaphorase

Localization of DPN diaphorase in the central nervous system of the rat differs from that of succinate dehydrogenase only in that the supporting structures, i.e. blood

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vessels, microglia and leptomeninges, react more intensely than the nerve cells. The patterns of staining of the remaining cells are similar although slightly less intense.

DPN-linked oxidases

WOLFGRAM and ROSE (1959) made histochemical investigations of malate, lactate and α-glycerophosphate dehydrogenases in the central nervous system of the adult rat and cat, using INT and Nitro-BT. They reported that formazan granules and crystals could be seen in the cells of the grey matter, and in astrocytes and oligodendrocytes, which they associated with the activity of specific dehydrogenases.

There are several objections to this conclusion. The use of INT at pH 8·2 is well within the range of activity of the so-called 'nothing' dehydrogenase (ZIMMERMANN and PEARSE, 1959), and much of the observed formazan was probably due to this non-specific effect. Probably more important than the above mentioned objection is the fact that WOLFGRAM and ROSE failed to recognise the need for high concentrations of co-enzyme (DPN) in the incubating medium (LOWRY, 1957). There is no evidence, in their paper, that exogenous DPN was used at all. In brain tissues, even if DPN is employed in the usual concentrations, the histochemical results for specific DPN-linked oxidases are weak or negative. This is due to the presence of large amounts of an enzyme (diphosphopyridine nucleotidase) which rapidly degrades both endogenous and exogenous DPN.

The action of this degrading enzyme and its inhibition by the use of nicotinamide were described by MANN and QUASTEL (1941), HANDLER and KLEIN (1942) and MCILWAIN and RODKNIGHT (1949). The very rapid rate of this degrading activity, and its completeness (75 per cent) in the central nervous system, were reported by GORE, IBBOTT and MCILWAIN (1950). The necessity for adding DPN and for its protection by the use of nicotinamide in studying DPN-linked dehydrogenases was demonstrated by the negative incubating media controls of this study. For all of these reasons, it is felt that the results reported by WOLFGRAM and ROSE were due to non-specific, non-enzymic, activity.

Microchemical methods

A series of microchemical investigations of enzymes in the central nervous system (including malate and lactate dehydrogenases) have been reported. These were based upon techniques and biochemical procedures modified or developed by LOWRY and his associates (LOWRY, 1953; ROBINS and SMITH, 1953; LOWRY, ROBERTS, LEINER, WU and FARR, 1954; LOWRY, ROBERTS, WU, HIXON and CRAWFORD, 1954; STROMINGER and LOWRY, 1955; LOWRY, ROBERTS and CHENG, 1956; ROBINS, ROBERTS, EYDT, LOWRY and SMITH, 1956; LOWRY, 1957; ROBINS *et al.*, 1957). Enzyme activities were estimated in successive tangentially cut layers of cerebral and cerebellar cortices and of subjacent white matter in the monkey, rabbit and rat. Selected tracts from the monkey and the rabbit, and the hypothalamus in the rabbit, were also investigated. In all the grey areas the activities of malate dehydrogenase were found to exceed those of lactate dehydrogenase by a factor of 2·5–4·7 (ROBINS and SMITH, 1953; STROMINGER and LOWRY, 1955; ROBINS, SMITH, EYDT and MCCAMAN, 1956; ROBINS, ROBERTS, EYDT, LOWRY and SMITH, 1956; KUHLMAN and LOWRY, 1956; SMITH *et al.*, 1957; ROBINS *et al.*, 1957; ROBINS *et al.*, 1958). There was no difference in the activities of the two enzymes in the subjacent white matter. With the

LOWRY methods significant differences in enzyme concentration have been demonstrated between the adjoining layers in cerebral iso and allocortex and in the cerebellar cortex. From the greater amount of activity in the molecular layer of the cerebellum and in certain layers of the cerebral cortex LOWRY (1957) concluded that the more active areas were those containing relatively more dendrites and neuropil and fewer cell bodies.

Comparison of microchemical and histochemical results

There is no readily apparent explanation for the difference in relative amounts of malate and lactate dehydrogenase recorded by the two methods. The reason for the lower enzymic activity in the outermost cerebellar and cerebral cortical layers demonstrated by histochemical methods, and the higher activities in these same layers shown by the microchemical methods, lies in the manner in which the conclusions are derived. The activities of the two enzymes are determined histochemically by a subjective evaluation of the intensities of staining of the adjacent layers. In the microchemical investigation the amount of enzyme activity per unit time is related to the unit dry weight of the layer studied. As suspected by ROBINS and SMITH (1953), the differences in activities in the various layers of the cerebellum can be related to the presence of many nuclei in the granular layer belonging to enzymically inactive cells. These contribute to the higher dry weight per unit area of the granular layer and thus to an apparently lower enzyme activity. A similar mechanism is probably responsible for the microchemical results in the cerebral cortex.

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Comparison of macrochemical and histochemical results

Comparison of the results of macrochemical methods with those of slide histochemistry are more difficult. KAWAKITA (1956) used homogenized rabbit kidney, liver and brain. With the kidney considered to represent 100 per cent succinate dehydrogenase activity, the activity in the liver was 75 per cent and in the brain 15 per cent. Within the brain the amount of enzyme activity was highest in the cerebellum, almost as high in the cerebrum and less in the brain stem and spinal cord. KAWAKITA also reported that the activity in the cerebral grey matter was 51–57 per cent of that of the white matter. Using homogenized rabbit brain and liver, and considering the latter as 100 per cent succinate dehydrogenase activity, EICHEL and SWANSON (1957) found that the activity in the brain varied between 65 and 117 per cent. With histochemical methods, liver succinate dehydrogenase activity was consistently greater than that of brain. Within the brain the activities of the various levels reported by KAWAKITA (1956) seem reasonable when unit areas are considered. The preponderant activity of succinate dehydrogenase in the cerebral white matter, however, is diametrically opposed to that seen with histochemical methods and cannot be explained.

In mitochondrial preparations of pig brain RINGLER and SINGER (1958) found about three times as much succinate as α-glycerophosphate dehydrogenase activity. SACKTOR, PACKER and ESTABROOK (1959), using mitochondrial preparations of rat brain, found α-glycerophosphate dehydrogenase to be the most active enzyme, followed in decreasing order by succinate dehydrogenase, DPN diaphorase and lactate dehydrogenase. Results derived from the literature by MCILWAIN (1959) indicated that the activity of malate dehydrogenase exceeded that of succinate dehydrogenase. An estimation of the rates of activity of the five oxidative enzymes recorded in this

histochemical study places the overall activities of succinate dehydrogenase and DPN diaphorase as approximately equal. Much less, but again approximately equal, are the activities of malate and lactate dehydrogenase. Least is the activity of α-glycerophosphate dehydrogenase.

The orders of activities and the localizations demonstrated in any of the methods must be accepted with some reservation. It must first be assumed that each reaction has been made under optimal conditions for the enzyme concerned. With the DPN-linked enzymes the assumption must also be made that the activity of the DPN degrading enzyme is adequately blocked so as to permit the maximal reaction to occur. It is possible, though unlikely, that the level of formazan deposition in the various cells of the central nervous system represents, to some extent, the varying amounts of the DPN-nucleotidase in these cells. It is more likely that diffuse, extracellular, nucleotidase activity lowers the available DPN concentration to the point at which an insufficient quantity is present in individual cells.

SUMMARY

The localization and comparative activities of five oxidative enzymes in rat brain have been studied by histochemical methods, using MTT and Nitro-BT as hydrogen acceptors.

The three DPN-linked enzymes (malate, lactate and α-glycerophosphate dehydrogenases) were found to require special conditions for their demonstration. Owing to the high DPN-nucleotidase activity of brain, positive reactions could only be obtained after blocking this enzyme with high concentrations of nicotinamide. The two remaining enzymes (succinate dehydrogenase, DPN diaphorase) required no special conditions and the addition of PMS was not found to be necessary, or helpful.

All five enzymes were present in the perikaryon and processes of all nerve cells and in the cytoplasm of cells lining the choroid plexuses, cervical central canal, and ventricles. They were present also in the cells of the pia-arachnoid, in blood vessels, and in microglia. Localization in astrocytes and oligodendrocytes was suspected but not proven.

Comparison of histochemical results with those of microchemical and macrochemical techniques could only be made with difficulty. There were, nevertheless, outstanding differences in the localization and relative quantities of the oxidative enzymes shown by histochemical and other methods. In cerebral and cerebellar cortex a possible explanation for these differences is offered.

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THE FATE OF INTRACISTERNALLY INJECTED TRACER PHOSPHATE IN THE RABBIT, NORMALLY AND AS INFLUENCED BY SULPHYDRYL REAGENTS

A KINETIC APPROACH TO THE MECHANISM OF THE BLOOD–BRAIN BARRIER

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ISOTOPIC tracer techniques have been used successfully during the past years for studying various anatomical, biochemical and physiological aspects of the permeability barrier system surrounding the central nervous system, commonly termed the 'blood–brain barrier'. Valuable information has thus become available concerning the embryonic and postnatal development of the blood–brain barrier, the rates of exchange of different ions and metabolites between the central nervous system and the rest of the body, the role of the cerebrospinal fluid (CSF) in brain metabolism, and several other problems (for reviews, see WAELSCH, 1955; BAKAY, 1956; HERLIN, 1956). With the aid of sub-arachnoidally administered radioactive phosphate it has also been possible to obtain a true estimate of the turnover rate of high-energy phosphate in the central nervous system (LINDBERG and ERNSTER, 1950) and thus to recognize the high-metabolic activity of this tissue.

Relatively little has so far been learnt about the actual physiological mechanism underlying the blood–brain barrier. Detailed kinetic studies of exchange processes taking place across the barrier—a conceivable way of approaching this problem—have been hampered by the fact that, because of technical difficulties, in most tracer experiments only a single sample of CSF could be obtained from one animal and thus no systematic time-study could be achieved.

The recent elaboration of a special surgical procedure for obtaining serial samples of CSF from the *cisterna magna* of one rabbit has rendered it possible to follow the disappearance of an intracisternally injected radioactive isotope from the CSF as a function of time (HERLIN, 1956). Such studies have been performed, using radioactive phosphate as a tracer, on normal rabbits, and on rabbits in which the barrier mechanism has been damaged experimentally by the intracisternal injection of a single dose of the sulphydryl reagent, *p*-chloromercuribenzoate (PCMB). It was found that the rates of exchange of phosphate between CSF and the rest of the body were different in the normal and in the PCMB-treated animals, not only on a simple quantitative basis, but also in a qualitative manner, as revealed by the shapes of the two time-curves. The present paper reports on an extension of these observations, with an attempt to evaluate more precisely the observed differences on a kinetic basis, and to assess their possible physiological implications regarding the mechanism of the blood–brain barrier.

EXPERIMENTAL

The surgical technique used in the present work has been described in detail previously (HERLIN 1956). The experimental procedure was as follows:

Albino rabbits (average weight 2·5 kg) were narcotized by the intravenous injection of 7 ml of 20% urethane solution per kg body-weight. The *membrana atlanto-occipitalis* was exposed, and from a tuberculine syringe, 0·1 ml physiological saline containing 0·05–0·1 mc 'carrier-free' ^{32}P-orthophosphate (purchased from the Atomic Energy Research Establishment, Harwell, England) was injected into the *cisterna magna*. In the experiments with sulphydryl reagents, the solution to be injected also contained the reagent, in a final concentration of 0·01 M. Samples of CSF were removed at intervals usually of 2, 5, 10, 15, 20, 30 and 40 min following the injection, using a self-filling micropipette of a volume of 2·66 μl. The samples were transferred directly to aluminium planchets covered with filterpaper, dried, and their content of ^{32}P was measured in a Geiger–Müller counter.

RESULTS AND DISCUSSION

Disappearance of intracisternally injected radioactive phosphate from the CSF-space. Table 1 shows the relative concentration of ^{32}P in the CSF in normal and PCMB-treated animals after various time intervals following the intracisternal injection of a single dose of ^{32}P-orthophosphate. The values are related percentage wise to the concentration of ^{32}P that was found in the CSF 2 minutes after the administration of the ^{32}P-orthophosphate. This time was considered as sufficient to allow an even distribution of the isotope in the CSF-space. This assumption was based on the previous finding (LINDBERG and ERNSTER, 1950) that subarachnoidally administered

TABLE 1.—DISAPPEARANCE OF INTRACISTERNALLY INJECTED TRACER PHOSPHATE FROM THE CSF-SPACE OF NORMAL AND PCMB-TREATED RABBITS

Time (min)		Relative content of ^{32}P in CSF	
After injection of tracer	After removal of first CSF-sample	CONTROLS (7 animals) (mean values ± S.D.)	PCMB-TREATED (5 animals) (mean values ± S.D.)
	(t)	(S_p)	(S_p)
2	0	100	100
5	3	30·2 ± 3·6	64·0 ± 3·1
10	8	19·2 ± 2·0	44·8 ± 4·0
15	13	13·1 ± 1·9	24·4 ± 2·1
20	18	10·7 ± 1·3	13·9 ± 0·8
30	28	7·2 ± 1·0	5·6 ± 0·4
40	38	6·0 ± 0·8	2·8 ± 0·3

^{32}P-orthophosphate was spread almost instantaneously within the brain tissue. The two groups of values differed significantly (Table 1). Moreover, the difference was of a qualitative rather than of a merely quantitative character, as indicated by the fact that the PCMB-values were *higher* than the controls at times below 18 minutes, and *lower* than the controls at times above 28 minutes; at 18 and 28 minutes, the two groups of values did not differ significantly. The nature of this difference will be clear from the following calculation.

Differential analysis of flow curves. The flow of a radioactive tracer metabolite from a given pool to an unlabelled depot can be described by the following equation:

$$-dS_p/dt = k \,.\, (S_p - S_r), \qquad (1)$$

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where S_p stands for the specific radioactivity (in the present case, $^{32}P/^{31}P$) of the radioactive pool (in the present case, the CSF), S_r for the specific radioactivity of the unlabelled depot to which the flow takes place, both at time t after the introduction of the tracer, and k stands for the velocity constant in terms of t^{-1}. If one assumed that the disappearance of the radioactive phosphate from the CSF proceeded according to a simple diffusion to the rest of the body, S_r would become negligible in comparison to S_p, since the phosphate content of the organism as a whole is much higher than that of the CSF. Equation (1) then takes the form,

$$-dS_p/dt = k \,.\, S_p. \tag{2}$$

Integrated over the interval, $t = 0$ to $t = t$, this equation gives

$$-\ln (S_p/S_0) = k \,.\, t, \tag{3}$$

or, in the explicit form,

$$S_p = S_0 \,.\, e^{-kt}, \tag{4}$$

where S_0 stands for the specific radioactivity of the labelled pool at $t = 0$; by equating this value to 100, S_p becomes the percent specific radioactivity of the pool at time t.

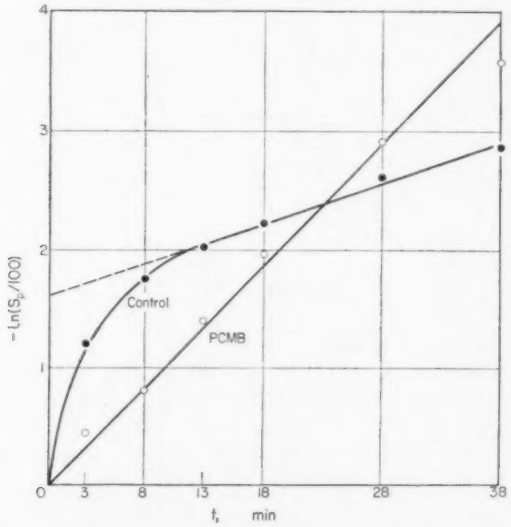

FIG. 1.—The experimental values from Table 1, as plotted according to Equation (3).

By assuming that the phosphate concentration of the CSF remains unchanged during the experiment, S_p is equal to the percent concentration of ^{32}P in the CSF at time t (cf. Table 1).

If the process here studied were a simple diffusion, it follows from Equation (3) that the logarithms of the experimental values shown in Table 1 should fit into a straight line when plotted against t, with k as the slope of the line. When this operation is made with the two groups of values (Fig. 1), the PCMB-values do, in fact, fit into a straight line; k can be estimated from the slope of this line to be 0·104 min^{-1}. The controls, on the other hand, describe a more complex curve, consisting of a steeply-rising initial phase, which levels off after about 4 minutes, and another, straight-line

component with a considerably more moderate slope. This biphasic character of the curve gives the impression of being the reflection of a two-step event, namely, a rapid equilibration of the ^{32}P between the radioactive pool and an unlabelled compartment of relatively small volume (e.g. a cell-layer, see below), followed by a relatively slow diffusion of ^{32}P from this intermediate compartment to the rest of the body. Such a process can be expressed by the following equation system:

$$-dS_p/dt = k_1 \,.\, (S_p - S_r) \tag{5}$$

$$dS_r/dt = k_1 \,.\, (S_p - S_r) - k_2 \,.\, S_r, \tag{6}$$

where S_p stands for the specific radioactivity of the CSF, S_r for the specific radioactivity of the 'intermediate compartment', both at time t, and k_1 and k_2 are the velocity constants, in t^{-1}, for the flow of ^{32}P from the CSF to the 'intermediate compartment', and from the latter to the rest of the body, respectively.

Computation of velocity constants. An attempt has been made to apply this equation system to the control group of values in Table 1, and to compute the velocity constants, k_1 and k_2. Solved with respect to S_p, Equations (5) and (6) give:

$$S_p = S_0(c \,.\, e^{-at} + (1 - c) \,.\, e^{-bt}), \tag{7}$$

where S_0 stands for the specific radioactivity of the CSF at zero-time (to be equated, as above, to 100), and where

$$c = C_p/(C_p + C_r), \tag{8}$$

C_p and C_r standing for the ^{31}P-content of the CSF and of the 'intermediate compartment', respectively, and

$$a = \tfrac{1}{2}(2k_1 + k_2 - \sqrt{4k_1^2 + k_2^2}), \tag{9}$$

$$b = \tfrac{1}{2}(2k_1 + k_2 + \sqrt{4k_1^2 + k_2^2}), \tag{10}$$

k_1 and k_2 being the velocity constants to be computed.

Since both k_1 and k_2 are positive values by definition, it follows that $b > a$, and consequently, that the e^{-bt} term of Equation (7) will decrease faster with increasing t than the e^{-at} term. Therefore, at sufficiently high values of t, the e^{-bt} term of Equation (7) becomes negligible in comparison to the e^{-at} term,* and the equation can be written in the simplified form

$$S_p = S_0 \,.\, c \,.\, e^{-at}, \tag{11}$$

or, in the logarithmic form,

$$-\ln (S_p/S_0) = a \,.\, t - \ln c. \tag{12}$$

Equation (12) represents a straight line, with $-\ln c$ as the intercept and a as the slope. These values can be estimated from the straight-line phase of the 'control' curve in Fig. 1 to $-\ln c = 1{\cdot}64$; $c = 0{\cdot}19$, and $a = 0{\cdot}03$. By substituting these values, together with the values of $t = 3$ and $S_p = 30{\cdot}2$ (from Table 1), into Equation (7), we obtain $b = 0{\cdot}62$. Substitution of $a = 0{\cdot}03$ and $b = 0{\cdot}62$ into Equations (9) and (10) gives $k_1 = 0{\cdot}293$ min^{-1} and $k_2 = 0{\cdot}063$ min^{-1}.

* With the values of $a = 0{\cdot}03$, $b = 0{\cdot}62$ and $c = 0{\cdot}19$, to be obtained below, it can be seen that, at $t = 13$, the $c \,.\, e^{-at}$ term will exceed the $(1 - c) \,.\, e^{-bt}$ term by a factor of 50. At higher t-values, obviously, the difference will become still higher.

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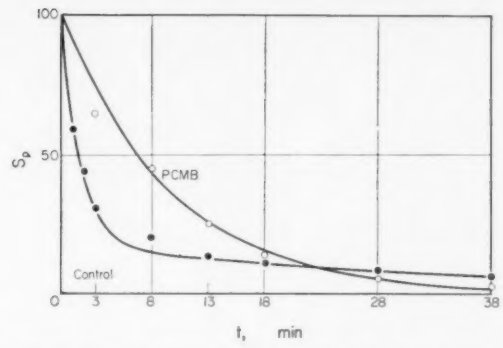

FIG. 2.—Comparison between theoretically derived lines and experimental points. The 'control' line corresponds to the equation, $S_p = 19 \cdot e^{-0.03t} + 81 \cdot e^{-0.62t}$, and the 'PCMB' line to the equation, $S_p = 100 \cdot e^{-0.104t}$. The experimental points are those taken from Table 1, except the two short-time points in the control group which come from a single experiment.

Fig. 2 compares the calculated curves,

$$S_p = 19 \cdot e^{-0.03t} + 81 \cdot e^{-0.62t} \tag{13}$$

and

$$S_p = 100 \cdot e^{-0.104t}, \tag{14}$$

derived for the control and the PCMB-treated animals, respectively, with the experimental values (Table 1). Equation (13) was obtained by substituting $a = 0{\cdot}03$, $b = 0{\cdot}62$, and $c = 0{\cdot}19$ into Equation (7), and Equation (14) was obtained by substituting $k = 0{\cdot}104$ into Equation (4). The experimental points in Fig. 2 also include, in the control group, two short-time values obtained in a single experiment. All the experimental values show satisfactory agreement with the theoretical curves.

Calculation of flow rates. The values derived above are consistent with the assumption that in the normal animal, the flow of phosphate between CSF and the rest of the body proceeds in two, kinetically distinct steps, one from the CSF to an 'intermediate compartment', and another, from this compartment to the rest of the body; the first step is relatively rapid, about 29·3 per cent (from the value of $k_1 = 0{\cdot}293$) of the total phosphate content of the CSF per minute, whereas the second is about five times slower, corresponding to a rate of exchange of about 6·3 per cent (from the value of $k_2 = 0{\cdot}063$) of the total content of phosphate of the CSF per minute. In the PCMB-treated animal, the two steps are no longer distinguishable kinetically, and the exchange process between CSF and the rest of the body assumes the character of a simple, passive diffusion; its rate corresponds to about 10·4 per cent (from the value of $k = 0{\cdot}104$) of the total content of phosphate of the CSF per minute.

To be able to compare the actual rates of phosphate exchange in the normal and PCMB-treated animals, it is necessary to know the actual phosphate contents of the CSF under the two sets of conditions. As reported previously (HERLIN, 1956), the normal animals had a mean content of phosphate in the CSF of 8·8 μg P per ml, whereas the PCMB-treated animals had a mean value of 36·8 μg P per ml. The latter value approaches the P-content of blood-plasma (cf. PALM, 1948). This seems further to support the concept that PCMB establishes a free diffusion of substances between CSF and the rest of the body. Furthermore, it has been found recently (ERNSTER, HERLIN and ZETTERSTRÖM, 1957) that PCMB also enhances the passage of serum

proteins and of bilirubin from the blood serum to the CSF. This would seem to indicate that the effect of intracisternally administered sulphydryl reagent in injuring the barrier mechanism is of a general nature rather than being specifically directed against the transport of phosphate.

In the case of the normal animal, the values of $k_1 = 0{\cdot}293$ min^{-1} and $k_2 = 0{\cdot}063$ min^{-1} mean that $8{\cdot}8 \times 0{\cdot}293 = 2{\cdot}6$ μg P are exchanged per minute and per ml CSF between CSF and the 'intermediate compartment', and $8{\cdot}8 \times 0{\cdot}063 = 0{\cdot}55$ μg P per minute and per ml CSF are exchanged between the 'intermediate compartment' and the rest of the body. In the PCMB-treated animal, an exchange rate of $36{\cdot}8 \times 0{\cdot}104 = 3{\cdot}85$ μg P per minute and per ml CSF is obtained. Thus, the rate of diffusion in the PCMB-treated animal is similar in magnitude to the rate of exchange between CSF

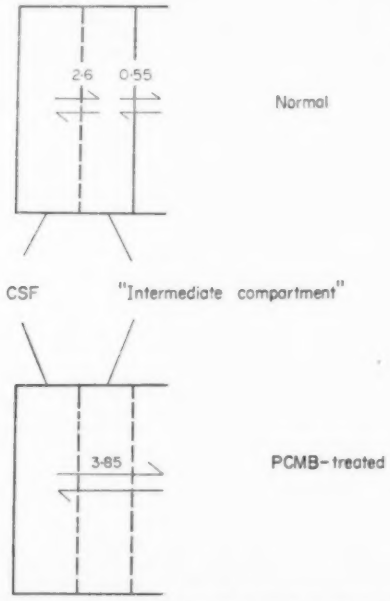

FIG. 3.—Schematic illustration of the concept that the 'distal' boundary of the 'intermediate compartment' constitutes the diffusion barrier proper for the diffusion of phosphate between CSF and the rest of the body, and of the effect of PCMB on this mechanism. The figures are flow rates of phosphate in terms of μg P/min per ml CSF.

and the 'intermediate compartment' in the normal animal, and it is considerably higher than the normal rate of exchange between the 'intermediate compartment' and the rest of the body. It therefore appears reasonable to conclude that it is the 'intermediate compartment', or more precisely, that it is the boundary of this compartment, located *distally* to the CSF-space, that constitutes the exchange barrier proper. This concept is schematically illustrated in Fig. 3.

Phosphate space and volume of the 'intermediate compartment'. From $c = C_p/(C_p + C_r) = 0{\cdot}19$ it follows that $C_r/C_p = 4{\cdot}3$, or, in other words, that the phosphate space of the 'intermediate compartment' (C_r) is 4·3 times larger than that of the CSF (C_p). However, if we assume that the 'intermediate compartment' is a cell-layer, it is to be expected that the ^{32}P-orthophosphate taken up into this layer is rapidly converted

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to the organic form, especially to high-energy phosphates, and that a rapid isotopic equilibration between these and the orthophosphate will occur. The rate of turnover of high-energy phosphates in brain tissue has previously been estimated to be 40 μg P per minute and per g brain (LINDBERG and ERNSTER, 1950). Provided that this value is valid for the cell-layer in question, this would mean that the rate of turnover of phosphate in the cell-layer would exceed the rate at which phosphate is exchanged with the CSF, 2·6 μg per minute and per ml CSF, by more than fifteen times, and, therefore, that the equilibration of the entering ^{32}P-orthophosphate with high-energy phosphates may be regarded as almost instantaneous. It is known, furthermore, that the orthophosphate content of brain tissue is about 20 μg P per g tissue, and the content of high-energy phosphates is about 30 μg P per g tissue (LINDBERG and ERNSTER, 1950). Together this makes 50 μg P per g tissue, that is, about 5–6 times more than the phosphate content of the CSF (8·8 μg P per ml CSF). Assuming, again, that these values, which have been determined in the brain tissue as a whole, are valid for the cell-layer here studied, this would mean that the cell-layer, with its phosphate space 4·3 times larger than that of the CSF, would have a volume similar to, or slightly less than, the CSF-volume. Considering that the CSF-volume of a rabbit is about 1·5 ml (HERLIN, 1956), this is still an unexpectedly high figure. For the sake of comparison it may be mentioned that the weight of the *plexus chorioidei* of the rabbit is of the order of 20 mg (wet weight). This seems to mean, either that the concentration of phosphate (inorganic + organic) is much higher in the cell layer here studied than in the brain as a whole, or that the barrier mechanism involves a larger array of anatomic elements, possibly including such areas as *arachnoideal villi*, *ependyme*, *leptomeninges*, *membrana limitans gliae*, etc. The fact, shown below, that intracisternally injected dinitrophenol does not influence the normal pattern of phosphate flow, would seem to render the first of these alternatives less probable. A better elucidation of this problem might be possible by using a metabolically inert tracer.

Neutralization of PCMB-effect by glutathione; effects of other agents. In order to ascertain whether or not the effect of PCMB in abolishing the barrier was due to its being a sulphydryl reagent, a control experiment was performed in which the PCMB-solution to be injected was mixed in advance with a slight excess (1·5 moles/mole) of glutathione, thus neutralizing its capacity to bind —SH-groups. As shown in Fig. 4, the logarithms of the percent radioactivity values obtained in a series of CSF-samples, when plotted against time, as described above (cf. Fig. 1), gave a biphasic curve of the shape typical for the normal animal. A further control experiment consisted in using another known sulphydryl reagent, N-ethylmaleinimide, which is otherwise chemically unrelated to PCMB. With this compound, a straight-line plot similar to that previously found with PCMB (cf. Fig. 1) was obtained (Fig. 5).

Among other metabolic inhibitors, the effect of 2:4-dinitrophenol is of special interest, since this compound uncouples cellular phosphorylation from respiration (LOOMIS and LIPMANN, 1948), and is known to inhibit certain active transport mechanisms in kidney tubuli (MUDGE and TAGGART, 1950; TAGGART and FORSTER, 1950). Two animals were injected intracisternally with a single dose of 5 μl of 0·02 M-dinitrophenol. One of the animals received ^{32}P-orthophosphate simultaneously with the dinitrophenol, and the other, 12 hr after the injection of the dinitrophenol. In the first animal, the concentration of dinitrophenol in the CSF, as measured 40 min after the injection, was $3{\cdot}7 \times 10^{-4}$ M; in the second animal, no dinitrophenol could be

detected in the CSF at the time of injection of ^{32}P-orthophosphate. Serial samples of CSF were removed from both animals, and the logarithms of the percent radioctivity values, taking the values obtained after 2 min as 100, were plotted against time in the way described above (cf. Fig. 1). In both instances biphasic, 'normal' curves were

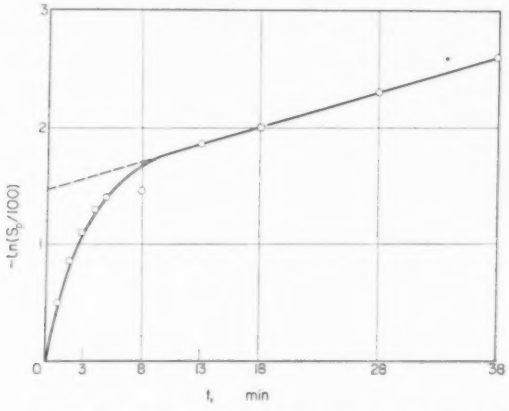

FIG. 4.—Neutralizing effect of glutathione on the action of PCMB on the blood–brain barrier. The plot resembles the normal plot in Fig. 1.

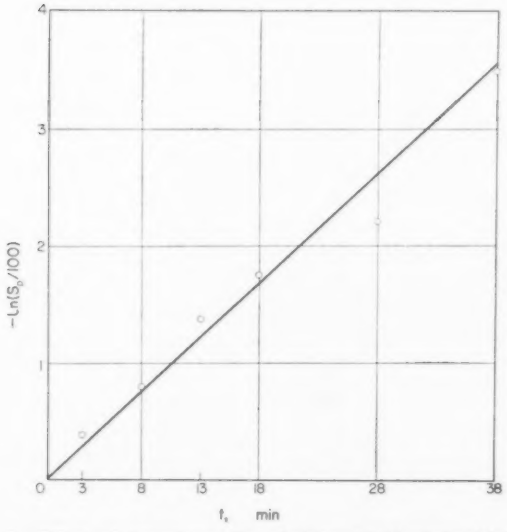

FIG. 5.—Effect of intracisternally administered N-ethylmaleinimide on the blood–brain barrier. The plot resembles the PCMB-plot in Fig. 1.

obtained (Fig. 6). This would seem to indicate that intracisternal administration of dinitrophenol, in a concentration which would be expected to cause a virtually complete suppression of the aerobic phosphorylation, has no influence on the barrier mechanism.

Whether this might be taken to mean that the barrier mechanism is independent of cellular energy-generating processes is however, questionable. First, it should be

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recalled that not all instances of oxidative phosphorylation are suppressed by dinitrophenol; the glycolytic phosphorylation (LYNEN and KOENIGSBERGER, 1951), for example, as well as the phosphorylation coupled to the substrate-level oxidation of α-ketoglutarate (HUNTER, 1951), are unaffected by this agent. Secondly, the possibility exists that phosphate-bond energy is required only for the *establishment* of a concentration gradient in a biological system, but not for its *maintenance*. Isolated kidney and liver mitochondria, for example, are able, both to concentrate certain ions

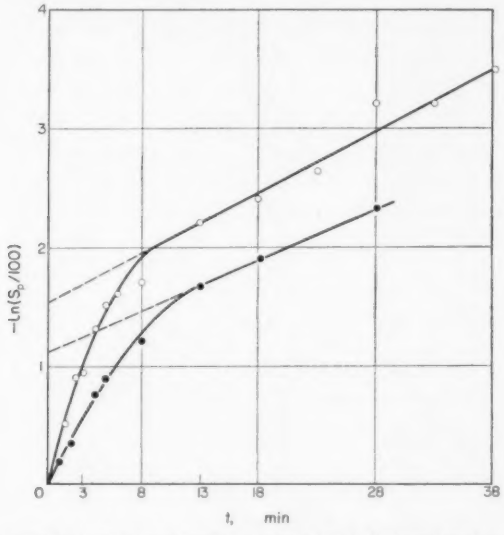

FIG. 6.—Lack of effect of intracisternally injected 2:4-dinitrophenol on the blood–brain barrier. The tracer phosphate was injected simultaneously with (circles), or 12 hr after (points), the injection of the dinitrophenol.

from a medium and to maintain a concentration of certain endogenous ions against a medium; the former requires an active oxidative phosphorylation and is inhibited by dinitrophenol, whereas the latter requires no oxidative phosphorylation and is unaffected by dinitrophenol (MACFARLANE and SPENCER, 1953; BARTLEY and DAVIES, 1954; and others. For review, see ERNSTER and LINDBERG, 1958).

It may be noteworthy, on the other hand, that PCMB has been reported (TAPLEY, 1956) to cause a drastic swelling of isolated liver mitochondria, thus destroying the lipoprotein structures probably responsible for the maintenance of concentration gradients. Provided that these aspects of mitochondrial permeability are of relevance to the level of the whole cell (cf. MITCHELL, 1959), the destructive effect of sulphydryl-inhibitors on the blood–brain barrier system may perhaps be sought primarily in the capacity of these agents to alter cellular lipoprotein structures rather than in their inhibitory action on specific enzyme reactions.

SUMMARY

'Carrier-free' ^{32}P-orthophosphate was injected into the *cisterna magna* of rabbits, and the decay of the ^{32}P-content of the cerebrospinal fluid (CSF), due to exchange of phosphate, was followed as a function of time. The decay-curve so obtained is consistent with the assumption that the radioactive phosphate on its way out from the

CSF space passes through an 'intermediate compartment' before reaching the extracerebrospinal space. The rate of exchange of phosphate between CSF and the 'intermediate compartment' has been estimated to be about 2·6 μg P/min per ml CSF, and between the 'intermediate compartment' and the rest of the body to be about 0·55 μg P/min per ml CSF. The phosphate space of the 'intermediate compartment' has been estimated to be about 4·3 times that of the CSF.

Administration of the sulphydryl reagent, *p*-chloromercuribenzoate (PCMB), along with the tracer phosphate, abolishes the biphasic character of the decay-curve, and the process assumes the character of a simple, passive diffusion between CSF and the rest of the body. The estimated rate of phosphate exchange is 3·85 μg P/min per ml CSF. Another sulphydryl reagent, N-ethylmaleinimide, produced a similar effect. The effect of PCMB could be nullified by the admixture of a slight excess of glutathione, which neutralizes the —SH-binding capacity of PCMB. 2:4-Dinitrophenol, injected intracisternally, did not alter the normal shape of the decay-curve.

The possible implications of these results for some anatomical and biochemical aspects of the blood–brain barrier have been discussed.

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CHOLINE BIOGENESIS—I

UTILIZATION OF [1:2-^{14}C] AMINOETHANOL IN CELL-FREE HOMOGENATES*

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THE classical investigations of choline biosynthesis by DU VIGNEAUD *et al.* (1941) demonstrated that the N-methyl groups of choline can be derived from methionine by transmethylation. However, STEKOL *et al.* (1955) found that dietary folic acid is required by rats for choline synthesis and postulated that, since folic acid is not necessary for transmethylation, two of the three methyl groups of choline must be synthesized *de novo* from such one-carbon donors as formate, formaldehyde, glycine, or serine. The apparent discrepancy between the findings of DU VIGNEAUD *et al.* and STEKOL *et al.* has not been resolved to date.

In 1941 STETTEN found that AE† provides the ethanolamine moiety of choline. Free AE and its methylated derivatives have been used since as acceptors of one-carbon units in numerous feeding experiments (STETTEN, 1942; DU VIGNEAUD *et al.*, 1946; WEISSBACH *et al.*, 1950; PILGERAM *et al.*, 1953; STEKOL *et al.*, 1955).

Previous attempts to study the synthesis of choline *in vitro* were hampered by the inability to find a cell-free system which would utilize AE as substrate. We prepared a cell-free enzyme extract from *S. cerevisiae* which utilizes [^{14}C]formate, [3-^{14}C]serine, [methyl-^{14}C]methionine, and radioactive AE in the synthesis of [^{14}C]choline. The cofactor requirements of this system and results obtained with it are described in this paper.

EXPERIMENTAL

Preparation of homogenates. A modification of a previously published method (ALEXANDER, 1957) was employed. Approximately 30 g of dry bakers' yeast (Fleischman) were suspended in 80 ml of 5% aqueous glycerol solution and stirred vigorously for 2 hr at room temperature. The resulting mixture was centrifuged at 2,000 **g** for 30 min to remove whole cells and cell debris. The supernatant solution was dialysed against three changes of 10 vol of distilled water over a 24-hr period at 2°. Clear, slightly opalescent homogenate was divided into 4 ml portions, and each portion placed in a 25 ml Erlenmeyer flask. Appropriate substrates and cofactors were added to the individual flasks at this point.

Incubation of homogenates. Cotton-plugged flasks were placed on a rotary swirling table for a 24-hr incubation at 29°. The table was rotated at the maximal safe speed because a high degree of aeration increased the yield. After the incubation was completed the reaction was stopped by addition of 4 ml of 95% ethanol and 2 min of boiling.

Isolation of choline. Portions of boiled homogenate were evaporated to a small volume. The lipids were extracted with chloroform-methanol (FOLCH *et al.*, 1951). The combined extracts were evaporated to dryness and hydrolysed with saturated $Ba(OH)_2$ at 100° for 3 hr. Excess $Ba(OH)_2$ was

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† Abbreviations used: Aminoethanol, AE; N-methylaminoethanol, MAE; N:N-dimethylaminoethanol, DMAE; adenosinetriphosphate, ATP; diphosphopyridinenucleotide, DPN.

removed with $(NH_4)_2CO_3$. The supernatant solution, including an added 5 mg of choline as carrier, was evaporated to dryness and the residue was dissolved in hot s-butanol. A fresh solution of ammonium reineckate in *sec.*-butanol (10 mg per ml) was added. The choline reineckate precipitate was collected by centrifugation and recrystallized from acetone by addition of water. Separate experiments were run to determine the fate of likely contaminants. S-adenosylmethionine was apparently decomposed during hydrolysis. Since the solution in which the reineckate was formed remained alkaline throughout the experiment, betaine reineckate, if present, remained in solution. When radioactive betaine was added to nonradioactive choline, less than 0·04 per cent of the impurity was retained after a standard treatment. Choline reineckate derived from [1:2-^{14}C]AE, recrystallized to constant activity, was redissolved in acetone and plancheted directly from solution for radioactivity measurements. Choline reineckate derived from radioactive one-carbon donors was treated further as described below.

Degradation of choline reineckate. After recrystallization, choline reineckate was placed in a three-neck flask and treated directly with 10% NaOH and 0·5% $KMnO_4$ as described by STREET *et al.* (1946). The resulting trimethylamine vapours were passed through two ammonia traps and absorbed by two HCl traps. After elimination of excess HCl by evaporation to dryness under a stream of nitrogen. the trimethylamine hydrochloride was dissolved in 95% ethanol and mixed with an equal volume of 0·5% ethanolic platinic chloride. The yellow precipitate was harvested by centrifugation, washed with ethanol, and dried. The trimethylamine chloroplatinate was suspended in 95% ethanol and spread on a planchet for counting.

Only negligible traces of radioactivity of [^{14}C]choline derived from [1:2-^{14}C]AE were present in the trimethylamine moiety. Conversely, 95–98 per cent of the radioactivity of [^{14}C]choline derived from radioactive one-carbon substrates, including [3-^{14}C]serine, was recovered as trimethylamine chloroplatinate. The conversion of [3-^{14}C]serine to [1:2-^{14}C]AE did not occur, therefore, in our enzyme system.

Paper chromatography. Portions of the homogenate were treated with 20% trichloroacetic acid (1·5 ml per 4 ml of homogenate) and recentrifuged. The clear supernatant solution was evaporated to dryness under nitrogen and digested with 2 ml of 6 N-HCl for 1 hr in a boiling water bath. After another centrifugation, 2 ml of 5·4 N-NaOH were added. The desalting was carried out in a test tube by addition of 1 g of Amberlite IR-4B and 1 g of Amberlite IRC-50 exchange resins in two separate cellophane tubes. The clear solution was concentrated under nitrogen and placed on sheets of Whatman No. 1 paper. Monodimensional ascending chromatograms were run preferentially, since it was possible to cut developed papers into strips and to determine the distribution of radioactivity in a scanning device, the RSC-5 Scanogram, Atomic Accessories, Inc. Standard samples were always run along with unknowns. Mixtures from all portions of any given experiment were run simultaneously on one paper. The active spots were cut out and the substances were eluted from them, reapplied to another sheet of paper, and rerun in a different solvent system. Determinations were made with the scanner and tested for correspondence with colour spots. The amino acids and aminoethanols were developed by spraying the paper with 0·5% ninhydrin solution in butanol (reagent supplied in a spray can by the Pierce Chemical Co., Rockford, Ill.). Choline was developed by the phosphomolybdic acid–stannous chloride method (LEVINE and CHARGAFF, 1951). The following solvent systems were employed: *iso*propanol–ammonia–water (40:1:12), phenol–water (4:1), and *n*-propanol–acetic acid–water (12:1:3). Total recovery of ^{14}C in the choline phosphomolybdates agreed closely with the recovery of ^{14}C in corresponding choline reineckates.

Materials. Radioactive substrates were purchased from Volk Radiochemical Company, except [methyl-^{14}C]betaine, which was kindly provided by Dr. D. SPRINSON of this Department. The purity of [1:2-^{14}C]AE was tested by ascending paper chromatography as described in the preceding section. It gave only one ^{14}C peak, proportional to the quantity applied to the paper. [3-^{14}C]Serine gave the same results. Oxidation of approximately 10 per cent of [methyl-^{14}C]methionine to a sulphoxide resulted in two radioactive spots. Sodium [^{14}C]formate was used directly without any attempt to establish its purity.

Folic acid, Leucovorin, and Aminopterin (4-aminopteroylglutamic acid) were gifts from the Lederle Division of American Cyanamid Company. ATP was purchased from Pabst Laboratories, DPN from Sigma Chemical Corporation, vitamin B_{12} from National Drug Company. AE, MAE, and DMAE were obtained from Eastman Kodak Company and distilled under reduced pressure prior to utilization. The reinecke salt used in choline isolation was prepared according to DAKIN

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(1943). A sample of S-adenosylmethionine was kindly provided by Dr. S. H. MUDD, National Institute of Mental Health, Bethesda, Maryland.

RESULTS

Utilization of [1:2-^{14}C]*AE and sodium* [^{14}C]*formate.* The cofactor requirements of the system for synthesis of [^{14}C]choline from AE and a one-carbon donor were established by measuring the recovery of ^{14}C in the choline fraction (Table 1). The

TABLE 1.—COFACTOR REQUIREMENTS IN CHOLINE SYNTHESIS

Description	Radioactive substrate					
	[1:2-^{14}C]AE			H^{14}COONa		
	Recovery of ^{14}C (counts/min)					
	CR†	TC	CP	CR	TC	CP
Complete system*	2,630	20	2,500	9,450	9,420	9,200
–HCOONa	1,440	—	1,300	—	—	—
–AE	—	—	—	3,870	3,820	3,700
–Folic acid	1,535	—	1,500	2,970	3,060	2,800
–DPN	1,820	—	1,700	5,700	5,550	5,400
–Homocysteine	1,295	—	1,300	1,755	1,745	1,700
–$MgCl_2$	830	—	750	3,460	3,050	3,300
–ATP	250	—	210	990	930	925

* Complete system: 0·4 mM-ATP, 0·4 mM-DPN, 0·8 mM-folic acid, 0·5 mM-DL-homocysteine, 10 mM-$MgCl_2$, and 0·125 mM-radioactive substrate in 4 ml homogenate; phosphate buffer, pH 7·0. Flasks with [1:2-^{14}C]AE received 0·6 mM-HCOONa, those with H^{14}COONa received 0·6 mM-AE.

† CR—choline reineckate; TC—trimethylamine chloroplatinate; CP—choline phosphomolybdate.

conversion involves activation of the substrates and reduction of the formate carbon to the methyl group level, a process that is repeated three times in the conversion of AE to a trimethyl derivative. Whether the reduction of formate occurs prior to or after a condensation with AE is a subject of a separate study. Because of the complexity of the over-all process, a wide variety of cofactors was necessary. A definite function can be assigned to some of them on the basis of previous experience. Thus, ATP, Mg^{++}, and DPN are necessary for the utilization of formate in the *de novo* synthesis of methyl groups. ATP can also serve as donor of the adenosyl moiety which is required in transmethylation (CANTONI, 1953). Homocysteine is required for methionine synthesis (DOCTOR *et al.*, 1957; NAKAO and GREENBERG, 1958). The requirement for folic acid, or more likely, its reduced derivative, tetrahydrofolic acid was further investigated with the aid of Aminopterin and Leucovorin (Table 2.) Competing one-carbon donors decreased the utilization of the carbon atom of formate in choline synthesis. S-adenosylmethionine was a stronger inhibitor than methionine. Betaine exerted no effect on [^{14}C]choline synthesis and glycine stimulated the synthesis of choline methyl groups from formate. This effect of glycine was noted consistently in sereval experiments.

The role of folic acid. Aminopterin inhibits the reduction of folic acid (FUTTERMAN, 1957). Since tetrahydrofolic acid rather than folic acid is the active principle, Aminopterin can prevent *de novo* methyl synthesis (DOCTOR *et al.*, 1957). It greatly reduced the incorporation of sodium [^{14}C]formate or [3-^{14}C]serine into [^{14}C]choline (Table 3). It lowered the yield of [^{14}C]choline from [1:2-^{14}C]AE because it prevented the

TABLE 2.—INHIBITION BY COMPETING ONE-CARBON DONORS

Additive	Radioactive substrate			
	[1:2-^{14}C]AE		$H^{14}COONa$	
	Recovery of ^{14}C in choline reineckate			
	counts/min	%	counts/min	%
—*	2,780	1·85	8,310	5·54
Methionine	—		1,175	
S-adenosylmethionine	—		0	
Serine	—		2,590	
Glycine	—		10,850	
Betaine	—		8,600	
Leucovorin	—		4,160	
Aminopterin	1,315		750	

* Complete system as described in Table 1. The concentration of additives: 12·5 mM except for Leucovorin and Aminopterin which were 0·8 mM. Flasks with Leucovorin and Aminopterin received no folic acid.

utilization of some one-carbon units. Transmethylation having been shown to be independent of folic acid, Aminopterin did not, as anticipated, inhibit the utilization of [methyl-^{14}C]methionine. By inhibiting the utilization of competing nonradioactive one-carbon donors and the resulting dilution of radiocholine, it increased the over-all yield of [^{14}C]choline from AE and preformed methyl groups.

TABLE 3.—FOLIC ACID REQUIREMENT IN CHOLINE SYNTHESIS

Source of ^{14}C	Folic acid	Amin-opterin	Recovery of ^{14}C in choline counts/min	%	Per cent change
[1:2-^{14}C] AE	+	−	2,400	1.6	
[1:2-^{14}C] AE	−	+	1,200	0·8	−50
$H^{14}COONa$	+	−	9,350	6·2	
$H^{14}COONa$	−	+	222	0·15	−98
DL-[3-^{14}C] Serine	+	−	10,830	7·2	
DL-[3-^{14}C] Serine	−	+	2,110	1·4	−81
L-[Methyl-^{14}C] methionine	+	−	29,400	19·6	
L-[Methyl-^{14}C] methionine	−	+	43,300	28·8	+47

Complete system as described in Table 1, except that Aminopterin replaced folic acid where indicated.

The recovery of ^{14}C in [^{14}C]choline from [methyl-^{14}C]methionine is much higher than could be accounted for by methylation of AE alone. It is obvious that [^{14}C]-choline is also synthesized from other precursors, probably, MAE and DMAE.

Vitamin B_{12} and choline synthesis. The role of vitamin B_{12} was investigated with the aid of three different radioactive one-carbon sources. Addition of minute amounts of the vitamin did not affect the incorporation of [methyl-^{14}C]methionine into [^{14}C]-choline to any appreciable extent, but it stimulated the incorporation of [^{14}C]formate and of the β-carbon atom of serine (Table 4). When the concentration of vitamin B_{12} was tripled no effect was obtained. Higher concentrations inhibited synthesis of [^{14}C]-choline, even from [3-^{14}C]serine. Several other reactions were investigated to establish

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whether or not the addition of vitamin B_{12} resulted in a general stimulation of metabolic activity. The synthesis of fatty acids and digitonin-precipitable steroids from sodium [1-^{14}C]acetate, transfer of the methyl group of [methyl-^{14}C]methionine to [28-^{14}C]ergosterol, and synthesis of [^{14}C]cystathionine from [3-^{14}C]serine and homocysteine were among the reactions tested. None showed any sensitivity to exogeneous vitamin B_{12}.

TABLE 4.—THE FUNCTION OF VITAMIN B_{12}

Source of ^{14}C	Vitamin B_{12}	Recovery of ^{14}C in choline counts/min	%
H^{14}COONa	—*	5,430	3·6
H^{14}COONa	+	8,980	6·0
DL-[3-^{14}C] Serine	—	6,600	4·4
DL-[3-^{14}C] Serine	+	11,950	7·9
L-[Methyl-^{14}C] methionine	—	30,500	20·3
L-[Methyl-^{14}C] methionine	+	31,480	21·0

* Complete system as described in Table 1. The concentration of vitamin B_{12}: 10 μg per flask.

Glycine effect. Addition of glycine, a one-carbon donor, did not decrease the ability of the enzyme system to utilize [^{14}C]formate in the synthesis of the methyl groups of choline. On the contrary, a definite stimulation was noted. Further experiments showed that this stimulation is not limited to formate, but extends to all one-carbon donors tested, including [methyl-^{14}C]methionine (Table 5). Doubling the concentration of glycine was sufficient to double the yield of [^{14}C]choline from [methyl-^{14}C]-

TABLE 5.—ROLE OF GLYCINE IN CHOLINE SYNTHESIS

Source of ^{14}C	μMoles glycine per flask	Recovery of ^{14}C in choline counts/min	%
H^{14}COONa	—	8,310	5·5
H^{14}COONa	100	10,850	7·2
DL-[3-^{14}C] Serine	—	9,350	6·2
DL-[3-^{14}C] Serine	100	12,600	8·4
L-[Methyl-^{14}C] methionine	—	18,200	12·1
L-[Methyl-^{14}C] methionine	1	37,300	24·8
L-[Methyl-^{14}C] methionine	10	34,800	23·2
L-[Methyl-^{14}C] methionine	100	31,300	20·9

Complete system as described in Table 1. Glycine concentration varied as shown.

methionine. The effect persisted even when the concentration of glycine was increased 100 times. The stimulation was caused specifically by glycine; addition of alanine, valine, or threonine did not affect the incorporation of [methyl-^{14}C]methionine into choline, and it severely decreased the incorporation of the β-carbon atom of serine (Table 6).

DISCUSSION

A yeast homogenate has been used in this study because homogenates of animal origin were incapable of utilizing [1 : 2-^{14}C]AE in choline synthesis. Mammalian

tissue homogenates did, however, utilize S-adenosylmethionine. Conclusions based on results obtained with mammalian preparations are apt to be misleading, since they obviously lacked certain enzymes present in intact animals and in tissue slices. It is impossible to determine the origin of all three methyl groups of choline in a homogenate which cannot utilize the most logical acceptor of the three methyl groups. The

TABLE 6.—EFFECT OF ALANINE, VALINE AND THREONINE

Source of ^{14}C	Additive	Recovery of ^{14}C in choline counts/min	%
DL-[3-^{14}C] Serine	—	7,700	5·1
DL-[3-^{14}C] Serine	Alanine	2,700	1·8
DL-[3-^{14}C] Serine	Valine	4,010	2·7
DL-[3-^{14}C] Serine	Threonine	3,100	2·1
L-[Methyl-^{14}C] methionine	—	19,750	13·2
L-[Methyl-^{14}C] methionine	Alanine	18,900	12·6
L-[Methyl-^{14}C] methionine	Valine	21,470	14·3
L-[Methyl-^{14}C] methionine	Threonine	17,130	11·4

Complete system as described in Table 1. Concentration of additives: 12·5 mM.

disadvantages of using a microbial extract are equally obvious. Utilization of data obtained in microbial preparations in explanation of animal phenomena is legitimate only if the data can be verified by or correlated with independent testing in the animal.

The preparation of a yeast homogenate is very simple and the stability of the enzymes is excellent. Temperature control is not critical. On the other hand, the wealth of needed cofactors, perhaps useful at first, quickly becomes a distinct disadvantage. Purification of the enzymes becomes imperative if further information into the nature of the processes is to be gained. Such a separation of the enzymes involved in choline synthesis is now in progress in this laboratory.

The utilization of AE in choline synthesis was first shown with the use of [^{15}N]AE in rat feeding experiments (STETTEN, 1941). The use of ^{14}C-labelled AE established the fact that AE provides the 2-carbon skeleton of choline (PILGERAM *et al.*, 1953). In rat liver slice experiments the latter investigators obtained up to 0·8 per cent yields of [^{14}C]choline from [1:2-^{14}C]AE (PILGERAM *et al.*, 1957). In our cell-free yeast homogenate yields of 1·6–1·8 per cent were obtained. Since no radioactivity was found in trimethylamine after degradation of [^{14}C]choline all must have resided in the aminoethanol moiety of choline. No conversion of AE via glycine (WEISSBACH and SPRINSON, 1953; PILGERAM *et al.*, 1957) and glyoxylate to formate (WEINHOUSE, 1955) took place. The incorporation of formate into the choline methyl groups was inhibited by competing one-carbon donors, methionine and serine. S-adenosylmethionine, apparently a more immediate form of methyl donor than methionine (CANTONI, 1953; STEKOL *et al.*, 1958) was much more inhibitory than free methionine. It is likely that formate may be incorporated into choline via S-adenosylmethionine without going through free methionine. Leucovorin, which contains an available one-carbon unit, inhibited the utilization of [^{14}C]formate, even though it was present in comparatively small amounts.

There is no agreement among investigators on the function of vitamin B_{12}. A requirement for this vitamin in the synthesis of the methyl moiety of methionine by

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cell-free extracts of a methionine-dependent strain of *E. coli* was reported (HELLEINER and WOODS, 1956). Vitamin B_{12} stimulated the reduction of formate or serine to the methyl group of choline in growing rats (ARNSTEIN, 1959). Other workers found that a deficiency of vitamin B_{12} in the diet of adult rats did not affect methionine synthesis and that addition of the vitamin to a methionine-methyl synthesizing system of amimal origin had no effect (STEKOL *et al.*, 1958). Recently, STEVENS and SAKAMI (1959) investigated the synthesis of methionine from formaldehyde in a fraction from pig liver and found that vitamin B_{12} improved somewhat the yield of [methyl-^{14}C]methionine in some experiments, but the effect was not reproducible.

The addition of vitamin B_{12} to our homogenate stimulated the incorporation of formate and serine-carbon into choline. It had no effect on the incorporation of the methyl carbon atom of methionine. This finding is in accord with the parallel results of ARNSTEIN (1959) in intact animals. ARNSTEIN concluded that vitamin B_{12} increases the reduction of formate or the β-carbon atom of serine to the methyl group level but does not affect transfer of one-carbon units at the same oxidation level.

The stimulatory effect of glycine, but not of other amino-acids of small molecular weight, is difficult to explain. Glycine and glyoxylate, tested among other compounds as electron donors in the reduction of folic acid by an enzyme system from *C. sticklandii*, were inactive (WRIGHT *et al.*, 1958). Since, in our hands, glycine stimulated the utilization of methionine as well as formate and serine, an explanation involving folic acid does not suffice. A similar effect of glycine was noted when it reversed somewhat the succinate inhibition of the conversion of [1:2-^{14}C]AE to choline in rat liver slices (PILGERAM, 1959). Since glycine increased the incorporation of all three one-carbon donors in our homogenate and since it was stimulatory in the utilization of AE as well, it must be involved in a step in choline synthesis which is common to all four of these substrates. There is at least one such step: the activation of AE as a one-carbon unit acceptor. This reaction has not been studied extensively because no purified enzyme preparation has been available for such a study. The effect of glycine may not be limited to AE but may include MAE and DMAE.

If we assume that all three methyl groups of choline arise by transmethylation, perhaps from S-adenosylmethionine, and even formate has to be reduced to a methyl group before it is transferred to AE, then glycine may be involved in the transfer of methyl groups. There is one finding in favour of the latter hypothesis: glycine stimulated not only the synthesis of choline but also of [28-^{14}C]ergosterol from one-carbon donors (ALEXANDER, 1960).

SUMMARY

A cell-free homogenate capable of utilizing aminoethanol in choline synthesis was prepared from yeast. A maximal yield of 1·8 per cent of [1:2-^{14}C]choline was obtained from [1:2-^{14}C]aminoethanol. The homogenate also synthesized [methyl-^{14}C]choline from aminoethanol and [^{14}C]formate, [3-^{14}C]serine or [methyl-^{14}C]methionine. The conditions for these transformations are described. Addition of vitamin B_{12} enhanced the incorporation of formate and of the β-carbon atom of serine, but not of methionine methyl into the methyl groups of choline. Glycine stimulated choline synthesis from all one-carbon donors tested.

Acknowledgement—The valuable assistance of Mrs. FLORENCE C. BRAND is gratefully acknowledged.

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CHOLINE BIOGENESIS—II

THE EFFECT OF HOMOCYSTEINE ON SERINE METABOLISM*

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THE β-carbon atom of serine can serve as a precursor of the methyl group of methionine (DOCTOR *et al.*, 1957; NAKAO and GREENBERG, 1958). This carbon atom has been found in the C–28 position of ergosterol and the N-methyl groups of choline synthesized in yeast homogenates (ALEXANDER and SCHWENK, 1958; ALEXANDER, 1959). Among the requirements for the utilization of serine in the synthesis of choline or ergosterol is homocysteine. In its absence, the β-carbon atom of serine is not converted to the methyl group. The function of homocysteine has not been adequately explained. The compound can obviously act as an acceptor of the one-carbon unit in the synthesis of methionine, but methionine (or its activated form) has not been established as an obligatory intermediate in the *de novo* synthesis of methyl groups of choline from serine, formaldehyde, or formate (Fig. 1). Alternatively, homocysteine

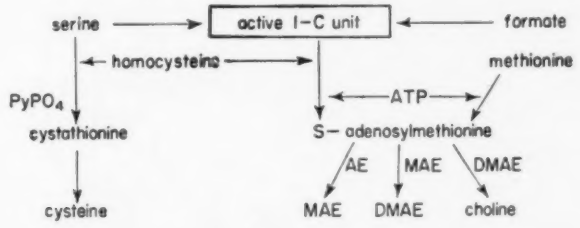

FIG. 1.—Proposed pathway of choline synthesis.

may be utilized in a more general manner, perhaps in synthesis of tetrahydrofolic acid required for the transfer and reduction of the β-carbon atom of serine. One form of active methionine, S-adenosylmethionine, has been identified both in animal tissues and in yeast (CANTONI and DURRELL, 1957; MUDD and CANTONI, 1958).

Addition of homocysteine to a crude yeast system, capable of synthesizing choline or ergosterol from serine, introduces certain problems. In the presence of homocysteine, serine can enter a competing reaction in which the entire molecule of serine condenses with homocysteine to form the amino acid, cystathionine (BINKLEY *et al.*, 1952). The latter amino-acid is not utilized as a source of one carbon units, but is cleaved, enzymically, to cysteine. Pyridoxal phosphate is required for the activity of the condensation and cleavage enzymes (Fig. 1). A further complication has been discovered recently by SELIM and GREENBERG (1959) who found that a purified rat

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liver enzyme responsible for the synthesis of cystathionine is also capable of deaminating serine and producing pyruvic acid and ammonia.

The present paper deals with the properties of a yeast homogenate which utilizes [3-C^{14}]serine as a donor of one-carbon units in the synthesis of choline and ergosterol and with the effect of homocysteine on these transformations.

EXPERIMENTAL

Homogenate: preparation and incubation. Dry bakers' yeast (Fleischman) was suspended in a 5% aqueous glycerol solution, agitated with a wire stirrer, centrifuged, and dialysed as described previously (ALEXANDER, 1960*a*). Each homogenate was divided into 4 ml portions and placed in 25 ml Erlenmeyer flasks. Substrates and cofactors were added and the cotton-plugged flasks were incubated with swirling on a rotating table at 29° for 24 hr. The incubation was stopped by addition of 4 ml of 95% ethanol and boiling for 2 min.

Isolation of end-products: ergosterol and choline. One-half of each boiled homogenate fraction was heated with 30% KOH (w/v) for 16 hr. Five mg of carrier ergosterol were added. The hydrolysates were treated with ether–petroleum ether (1:1). The extracts were washed with 10% aqueous KOH and evaporated to dryness. The nonsaponifiable fraction was treated with digitonin and free sterols were recovered after cleavage of digitonides with pyridine (SCHWENK *et al.*, 1955).

The other half of each fraction was evaporated to small volume and the lipids were extracted and hydrolysed. After addition of 5 mg of choline chloride carrier, choline was isolated as reineckate. The reineckate was degraded to trimethylamine and the latter precipitated as chloroplatinate (ALEXANDER, 1960*a*). In a typical experiment 97 per cent of the total radioactivity of choline reineckate was recovered in the trimethylamine chloroplatinate.

As before, portions of the homogenate were chromatographed on Whatman No. 1 paper in several solvent systems. The positions of aminoethanols and amino acids were determined by scanning the paper for radioactivity and correspondence with colour spots. After elution, the total radioactivity of choline phosphomolybdate was compared to the total radioactivity of choline reineckate and trimethylamine chloroplatinate derived from it. The yield of ^{14}C in eluted choline phosphomolybdate was 5–10 per cent smaller than the yield of ^{14}C in choline reineckate.

Materials. DL-[3-^{14}C]Serine was purchased from Volk Radiochemical Company. ATP was obtained from Pabst laboratories, DPN from Sigma Chemical Corporation. Samples of folic acid and Aminopterin were a gift from The Lederle Division of The American Cyanamid Company. Vitamin B_{12} was bought from The National Drug Company. AE*, MAE, and DMAE were bought from Eastman Kodak Company and distilled under reduced pressure prior to utilization. The reinecke salt used in choline isolation was prepared according to DAKIN (1943).

RESULTS

Cofactor requirements. Many portions of a homogenate were incubated simultaneously, with and without various additives to establish the cofactor requirements of the system (Table 1). ATP, folic acid, homocysteine, vitamin B_{12}, and Mg^{++} were necessary for efficient utilization of the β-carbon atom of serine in choline biosynthesis. The same cofactors were required in the synthesis of [28-^{14}C]ergosterol.

Another homogenate was incubated with homocysteine, cysteine, or reduced glutathione (Table 2). Low concentrations of homocysteine favoured the synthesis of [^{14}C]choline. The other thiols were without effect. Aminopterin was used as a tetrahydrofolic acid inhibitor. As expected, it was inhibitory, but the fact that inhibition was incomplete suggested that a large pool of tetrahydrofolic acid is present in the yeast homogenate. Low concentrations of homocysteine reversed somewhat the Aminopterin inhibition.

* Abbreviations used: Aminoethanol, AE; N-methylaminoethanol, MAE; N:N-dimethylaminoethanol, DMAE; adenosinetriphosphate, ATP; and diphosphopyridinenucleotide, DPN.

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TABLE 1.—COFACTORS IN THE UTILIZATION OF DL-[3-^{14}C] SERINE AS A ONE-CARBON DONOR

Description	Recovery of ^{14}C (counts/min)	
	Choline Reineckate	Ergosterol
Complete system*	9,950†	2,250
–DPN	9,700	2,170
–Vitamin B_{12}	5,500	1,150
–Folic acid	1,125	125
–Homocysteine	475	375
–ATP	380	50
–$MgCl_2$	60	225
–Aminoethanol	7,500	5,000
–Pyridoxal phosphate	10,400	2,575

* Complete system: 4 ml of homogenate, pH 7·0, 0·4 mM-ATP, 0·4 mM-DPN, 0·8 mM-folic acid, 0·5 mM-DL-homocysteine, 0·6 mM-AE, 0·8 mM-pyridoxal phosphate, 0·6 mM-DL-[3-^{14}C] serine (0·5 μC), 10 mM-$MgCl_2$, and 10 μg vitamin B_{12}.

† The recovery of ^{14}C in trimethylamine chloroplatinate: 9,650 counts/min (97 per cent); in choline phosphomolybdate eluted from paper = 9,260 counts/min (93 per cent).

Influence of various additives. Competing one-carbon donors, formate and methionine, inhibited utilization of the β-carbon atom of serine (Table 3). Formaldehyde which is not efficiently utilized by this system (ALEXANDER and SCHWENK, 1958) had a negligible effect, as did methanol. Glycine stimulated methyl group synthesis. A large excess of homocysteine inhibited serine utilization in methyl group synthesis. The

TABLE 2.—ROLE OF THIOLS IN METHYL GROUP SYNTHESIS

Additive	Concentration (mM)	Recovery of ^{14}C in choline reineckate (counts/min)
—*	—	400
Homocysteine	0·5	6,700
Homocysteine + Aminopterin	0·5 + 0·8	840
Homocysteine + Aminopterin	5·0 + 0·8	80
Aminopterin	0·8	350
Reduced glutathione	0·5	375
Cysteine	0·5	430

* Complete system: 4 ml of homogenate, ATP, AE, [3-^{14}C]serine, $MgCl_2$, and vitamin B_{12} as in Table 1. Folic acid was present in flasks without Aminopterin.

TABLE 3.—INFLUENCE OF COMPETING ONE-CARBON DONORS

Additive	Recovery of ^{14}C (counts/min)	
	Choline reineckate	Ergosterol
—*	6,500	700
Methionine	700	220
Sodium formate	650	290
Choline hydrate	—	280
Formaldehyde	4,900	650
Methanol	5,990	730
Glycine	8,800	1,000
Cystathionine	6,130	675

* Complete system: 4 ml of homogenate and cofactors described in Table 2. Concentration of additives: 1·2 mM.

role of homocysteine was investigated further. No effect was observed when cystathionine was added to the system.

The relative yields of choline and ergosterol could be manipulated by the addition of AE. AE caused a 50 per cent drop in sterol radioactivity (Table 1). Choline synthesis was stimulated by AE and MAE at low concentration, inhibited by all three at high concentrations (Table 4).

TABLE 4.—DERIVATIVES OF AMINOETHANOL AS PRECURSORS OF CHOLINE

Additive	Recovery of ^{14}C in choline reineckate (counts/min)			
	Amount of additive (m-moles per m-mole of [3-^{14}C] serine)			
	0	1	10	100
—*	3,640	—	—	—
AE	—	8,940	5,700	1,010
MAE	—	5,950	3,990	530
DMAE	—	2,780	1,980	190

* Complete system: 4 ml of homogenate and cofactors as described in Table 2.

Since addition of excess homocysteine resulted in lowered incorporation of the β-carbon of serine into methyl groups, it was assumed that homocysteine diverts the β-carbon from methyl group synthesis to the synthesis of cystathionine. This was confirmed in a series of experiments in which the radioactivity incorporated into cystathionine and cysteine increased in proportion to the concentration of homocysteine in the system (Fig. 2). The pattern of choline synthesis was more complex; after a small initial drop there was a sharp increase, followed by a steady, gradual decrease. When the concentration of homocysteine was increased to more than twice that of [3-^{14}C]serine there was a gradual decrease of radioactivity in choline, corresponding roughly to the increase in radioactivity of cystathionine and cysteine. The variations in choline radioactivity at low homocysteine concentration were observed consistently in three separate experiments.

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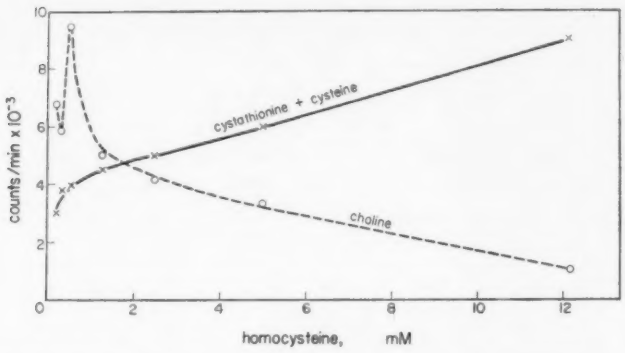


FIG. 2.—Role of homocysteine in serine metabolism. Portions of homogenate as described in Table 2 were used except for homocysteine which varied as shown.

DISCUSSION

The cell-free yeast homogenate used in this work was used previously in a study of the origin of carbon-28 of ergosterol (ALEXANDER and SCHWENK, 1958). It was noted then that up to 5 per cent of added [3-^{14}C]serine is utilized in the synthesis of the methyl group of ergosterol. In the present study the cofactor requirements of the system have been defined. Vitamin B_{12} was found to enhance the yields considerably. AE was added to increase choline synthesis which was not measured previously. Without exogeneous AE less than 5 per cent of the ^{14}C was recovered in [^{14}C]choline. Addition of the AE increased the [^{14}C]choline yield to 13·3 per cent. The synthesis of C-methyl and N-methyl groups is probably mediated by different sets of enzymes but the cofactor requirements are, at this preliminary stage, identical.

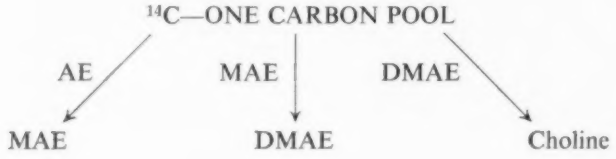
Another example of the use of [3-^{14}C]serine as a source of a C-methyl moiety in a microbial system has been found in the biosynthesis of citrinin by *Penicillium citrinum* (SCHWENK *et al.*, 1958). The mechanisms of ergosterol and citrinin syntheses appear to be similar: A one-carbon atom moiety, which can be derived from formate, serine, or methionine is attached to a carbon skeleton built from two-carbon units originating in acetic acid. The details of these transformations are still in doubt. Isolation of individual enzyme components of the system, now in progress, will enable us better to understand their mechanisms. ELWYN *et al.*, (1955) have established that both hydrogen atoms attached to the β-carbon atom of serine remain on this carbon atom during a *de novo* methyl synthesis. On the other hand, formaldehyde lost one of its hydrogen atoms during the process (LOWY *et al.*, 1956).

Our yeast system, unlike the mammalian systems of DOCTOR *et al.*, (1957), NAKAO and GREENBERG (1958), and STEVENS and SAKAMI (1959), does not have the ability to utilize [^{14}C]formaldehyde. Formaldehyde, therefore, did not dilute the one-carbon moiety derived from radioactive serine. Methionine and sodium formate contributed to the one-carbon pool. Addition of nonradioactive choline decreased the yield of the ^{14}C-labelled methyl group of ergosterol, even though methyl-[^{14}C]betaine is not utilized by this system in the synthesis of either choline or ergosterol (ALEXANDER, 1960*b*). A type of feedback mechanism may operate in this case.

The increased yield of [^{14}C]choline on addition of AE or MAE is not due to sparing

of [3-^{14}C]serine through a suppression of its decarboxylation. There is no evidence to show that any [3-^{14}C]serine is converted to AE in our yeast system. All the radioactivity of [3-^{14}C]serine incorporated into [^{14}C]choline resided in the N-methyl groups and no significant amounts of ^{14}C were in the AE moiety. Such sparing effect of serine would, moreover, be expected to enhance also the recovery of ^{14}C in [28-^{14}C]ergosterol, contrary to the experimental data.

The inhibition of [^{14}C]choline synthesis by high concentrations of AE and MAE may be due to their ability to accept all the available ^{14}C units from the pool, so that there is little left for methylating DMAE to choline.



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For example, an excess of AE might lead to an increased quantity of MAE, use up a disproportionate amount of radioactive one-carbon units, and thus lead to a diminished yield of [^{14}C] choline.

Unlike AE and MAE, DMAE decreased the synthesis of [^{14}C]choline from [3-^{14}C]-serine even at low concentrations. Were the concentration of the final methylating enzyme the limiting factor in choline synthesis, excess of DMAE might well lead to synthesis of [^{14}C]choline of low specific activity, with the molecular species containing only one radiocarbon predominating. It is also possible that DMAE undergoes a form of disproportionation in which some of its molecules provide methyl groups to the methyl pool whereas other molecules are being methylated to choline. A large excess of DMAE would noticeably dilute the ^{14}C-methyl pool and therefore decrease the total recovery of ^{14}C in choline.

Homocysteine is deemed essential in the *de novo* synthesis of methyl groups from serine. It serves as an acceptor of one-carbon units in the synthesis of methionine. Its function in the synthesis of choline or ergosterol from serine is not clear unless one accepts methionine or S-adenosylmethionine as obligatory intermediates. Optimum [^{14}C]choline synthesis in yeast homogenate resulted when the amount of DL-homocysteine in the system was twice as high as the amount of DL-[^{14}C]serine. It is apparent that homocysteine reacted with the radioactive one-carbon unit arising from serine and the resulting complex was used in the synthesis of methyl groups. There are other possible functions of homocysteine. Thiols may activate enzymes through formation of complexes with heavy metals and removal of inhibiting radicals. They may serve as reducing agents. For example, the reduction of folic acid to dihydrofolic acid by *A. sticklandii* requires reduced CoA at substrate level rather than DPNH or TPNH. BAL or cysteine, but not homocysteine, can spare CoASH (WRIGHT *et al.*, 1958). In my experiments the requirement was specific for homocysteine.

Increased amounts of homocysteine led to a decrease in choline synthesis. Addition of a large excess of homocysteine diverted an increased proportion of serine toward cystathionine synthesis and thus prevented any further disassociation of serine into glycine and the one-carbon precursor of methyl groups.

SUMMARY

A yeast homogenate, utilizing the β-carbon atom of [3-^{14}C]serine in the synthesis of methyl groups of choline and ergosterol, required ATP, folic acid, homocysteine, vitamin B_{12}, and Mg^{++} for optimum activity. Aminoethanol increased the incorporation of radioactivity into choline at the expense of ergosterol. Competing one-carbon donors inhibited serine utilization; glycine enhanced the synthesis of [^{14}C]choline and [^{14}C]ergosterol. Homocysteine at low concentrations also enhanced choline synthesis, but at high concentrations inhibited choline synthesis and stimulated synthesis of cystathionine.

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CHOLINE BIOGENESIS—III

THE ORIGIN OF THE THREE METHYL GROUPS*

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THE classical studies of DU VIGNEAUD *et al.* (1941) and KELLER *et al.* (1949) demonstrated that in intact rats the methyl groups of choline can be derived from methionine by transmethylation. Subsequently, STEKOL *et al.* (1955) found that folic acid stimulates choline synthesis and postulated that in normal rats well supplied with cofactors "choline is not synthesized . . . by transfer of three methyl groups of methionine to aminoethanol but by transfer of one methyl group of methionine to dimethylaminoethanol as the direct acceptor". Since folic acid is not involved in simple transmethylation but is essential in methyl neogenesis, STEKOL *et al.* and others after them assumed that the two methyl groups of dimethylaminoethanol are synthesized *de novo* in the organism from such sources of one-carbon units as formate, formaldehyde, or serine. Further investigation in intact animals as well as in microbial and mammalian homogenates failed to resolve the question of the origin of the three methyl groups of choline (STEKOL *et al.*, 1958; GREENBERG and FORAND, 1959; WOLF and NYC, 1959*a*). An enzyme system prepared from *S. cerevisiae* has been used in the present study of choline synthesis from AE†, MAE, and DMAE with [methyl-^{14}C]methionine, [3-^{14}C]-serine, and [^{14}C]formate as sources of the methyl group carbon atom.

EXPERIMENTAL

Preparation and incubation of homogenates. Dry bakers' yeast (Fleischman) was suspended in a 5% aqueous glycerol solution, agitated with a wire stirrer, centrifuged, and dialysed as described previously (ALEXANDER, 1960*b*). The enzyme homogenate was further centrifuged at 0° and 105,000 g for 30 min. The supernatant solution was made up to 80 ml, placed in two 250 ml Erlenmeyer flasks and preincubated at room temperature. After 16 hours, the solutions were combined, shaken, and divided into 4 ml portions in 25 ml Erlenmeyer flasks. The cofactors described previously and radioactive and nonradioactive substrates were added to the individual flasks. Incubation was carried out at 29° for 24 hrs and stopped by addition of 4 ml of 95% ethanol and boiling for 2 min. Under the experimental conditions, this homogenate did not synthesize proteins or utilize glucose. It did not synthesize cysteine. However, it still consisted of a very complex mixture of enzymes.

Isolation of choline. Each portion of the homogenate was divided in two. Choline was isolated from the first half with reinecke salt, after addition of carrier. Choline reineckate, recrystallized to constant specific activity, was degraded to trimethylamine (ALEXANDER, 1960*b*). Trimethylamine was precipitated as chloroplatinate, and plated out from a suspension in ethanol. The radioactivity was determined in a flow-gas counter. Specific radioactivity was calculated by dividing the total radioactivity in trimethylamine chloroplatinate by the weight of choline determined in the second half of each portion of homogenate by a modification of the micro method of SPERRY and BRAND

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† Abbreviations used: Aminoethanol, AE; N-methylaminoethanol, MAE; N : N-dimethylaminoethanol, DMAE; adenosinetriphosphate, ATP; diphosphopyridinenucleotide, DPN.

(1948). All values represent an average of 4 separate portions of one homogenate. Paper chromotography was used, as before (ALEXANDER, 1960*b*), to confirm the purity of the choline fraction.

Materials. Radioactive compounds were purchased from Volk Radiochemical Company, except for [methyl-^{14}C]betaine which was kindly provided by Dr. D. SPRINSON of this Department. Standard aminoacids were purchased from the California Corporation for Biological Research; vitamin B_{12} from the National Drug Company. Aminopterin (4-aminopteroyl glutamic acid) and folic acid were a gift from the Lederle division of the American Cyanamid Corporation. AE, MAE, and DMAE were purchased from the Eastman Kodak Co. and distilled under reduced pressure prior to use.

RESULTS

Comparison of carbon-14 donors. Several radioactive one-carbon donors were tested in separate portions of one yeast homogenate. AE or its methylated derivatives served as substrates. Previously determined adequate amounts of cofactors were supplied to the system. [Methyl-^{14}C]betaine was not utilized in synthesis of choline

TABLE 1.—ONE-CARBON DONORS AS PRECURSORS OF CHOLINE

Source of ^{14}C	Counts/min per μg		
	Substrate		
	AE	MAE	DMAE
Sodium [^{14}C] formate	630	405	191
DL-[3-^{14}C] Serine	1,071	678	318
L-[Methyl-^{14}C] methionine	4,670	3,110	1,930
[Methyl-^{14}C] betaine	10	2	12

Complete system—4 ml of yeast homogenate, pH 7; 0·4 mM-ATP; 0·8 mM-folic acid; 0·5 mM-DL-homocysteine (free base); 0·25 mM-AE, MAE or DMAE as indicated, 0.0625 mM-^{14}C donor (0·5 μC per flask), 10 mM-$MgCl_2$, and 10 μg vitamin B_{12}.

(Table 1). Among the compounds which were utilized, [methyl-^{14}C]methionine was by far the most efficient source of ^{14}C for [methyl-^{14}C] choline. It was the preferred intermediate, regardless of the nature of the acceptor of one-carbon units. We have repeated the experiment several times with [methyl-^{14}C]methionine and found that [^{14}C]choline from DMAE is slightly more than one-third as active as [^{14}C]choline from AE.

Role of Aminopterin in choline synthesis. Portions of a homogenate were incubated with and without the addition of Aminopterin. [Methyl-^{14}C]methionine and AE, MAE, or DMAE were used as substrates. Aminopterin, a folic acid inhibitor, prevented dilution of radiocholine by eliminating *de novo* synthesis of choline from nonradioactive precursors (Table 2). The same amount of radiomethionine was used in Aminopterin flasks as in control flasks. The total amount of radioactivity recovered in the choline fraction was approximately the same in both cases. The increase in specific activity of [^{14}C]choline is due to the elimination of a diluting synthesis of choline from nonradioactive precursors. This increase is directly proportional to the contribution of the eliminated process. Because less choline was synthesized in the system in the presence of Aminopterin, specific radioactivity of newly synthesized [methyl-^{14}C]choline measured in counts/min per μg was almost three times higher in flasks with Aminopterin than in controls (Table 2). This increase indicated that

approximately 62% of choline in control flasks was due to *de novo* synthesis of methyl groups.

The assumption that only the third methyl group of choline arises from methionine by transmethylation implies that Aminopterin inhibits [^{14}C]choline synthesis from [methyl-^{14}C]methionine and AE or MAE and permits synthesis only from DMAE. However, Aminopterin was not inhibitory in the case of AE or MAE; it had a stimulatory effect whether the addition of one, two, or three methyl groups was necessary to obtain [methyl-^{14}C]choline. In homogenates rich in folic acid as well as in homogenates to which Aminopterin was added, the specific activity of choline from AE was up to three times the specific activity of choline from DMAE.

TABLE 2.—INHIBITION OF *de novo* SYNTHESIS OF METHYL GROUPS BY AMINOPTERIN

Substrate	Recovery as [methyl-^{14}C] choline		Indicated dilution due to nonradioactive choline*
	Control counts/min/μg	Aminopterin counts/min/μg	%
DMAE	1,904	5,240	63·7
MAE	3,648	10,200	64·2
AE	5,280	12,740	57·8

Same cofactors as in Table 1., except that Aminopterin replaced folic acid when indicated. [Methyl-^{14}C]methionine was the source of ^{14}C.

$$\text{* Indicated dilution} = \frac{\text{counts/min/}\mu\text{g}_{\text{Aminopterin}} - \text{counts/min/}\mu\text{g}_{\text{control}}}{\text{counts/min/}\mu\text{g}_{\text{Aminopterin}}} \times 100.$$

For explanation see text.

DISCUSSION

The cell-free yeast homogenate utilizes AE as well as its methylated derivatives in choline synthesis. It is capable of transmethylation and of *de novo* methyl synthesis from formate or the β-carbon atom of serine. It is of use, therefore, in an investigation of the origin of the three methyl groups of choline.

Folic acid stimulates choline synthesis in intact rats (STEKOL *et al.*, 1955). Mammalian tissue homogenates do not utilize AE in choline synthesis (PILGERAM, 1959), while DMAE is an efficient methyl acceptor under similar conditions (STEKOL *et al.*, 1958). The conclusion drawn from these facts was that the 'first two' methyl groups of choline (*i.e.* the two methyl groups of DMAE) are derived from formate, formaldehyde, or serine, while the third methyl group is derived from methionine.

The data presented here do not support this conclusion. An alternative hypothesis which accounts for the available information is suggested here. The first two methyl groups of choline need not be derived exclusively by *de novo* synthesis nor the third methyl group exclusively from methionine. Instead, approximately one-third of each methyl group may be derived from methionine and two-thirds through *de novo* synthesis.

Direct synthesis of methyl groups on aminoethanol (*i.e.* reduction of N-formylaminoethanol to N-methylaminoethanol) has never been shown. Homocysteine, a precursor of S-adenosylmethionine, is essential in choline synthesis from AE and formate or serine (ALEXANDER, 1960*a*). The requirements for synthesis of methyl

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groups of choline from [3-^{14}C]serine are identical with the requirements for methionine synthesis followed by transmethylation; inhibition of transmethylation limits synthesis of choline from [3-^{14}C]serine (ALEXANDER, 1960*a*). Addition of S-adenosylmethionine completely eliminates choline synthesis from serine or formate (ALEXANDER, 1960*b*). [Methyl-^{14}C]choline synthesized from AE is almost three times as radioactive as choline synthesized from DMAE. Therefore, S-adenosylmethionine or a similar 'active methionine', synthesized partly by activation of exogenous methionine and partly by *de novo* process from homocysteine and formate or serine, is a likely intermediate in the synthesis of methyl groups of choline not only from methionine but also from formate or serine. The 3 : 2 : 1 ratio obtained regardless of the original source of the methyl group carbon atom (Table 3) indicates that all three methyl groups arise in a similar manner from the same one-carbon pool. It is this ultimate one-carbon source (S-adenosylmethionine?) which is derived partly from formate or serine and partly from methionine.

TABLE 3.—ROLE OF AMINOETHANOL AND ITS DERIVATIVES IN CHOLINE BIOGENESIS

Substrate	Recovery as [methyl-^{14}C]choline				
	[^{14}C]formate	[3-^{14}C]serine		[Methyl-^{14}C]methionine	
DMAE	100*	100*	100†	100*	100‡
MAE	64	67	63	67	67
AE	31	31	30	41	35

* Data based on information in Table 1.
† Data based on information in Table 4 of the preceding paper.
‡ Data based on information in Table 2.
Since DMAE values were used as a base the horizontal columns are not comparable.

The ratio of methyl groups derived from [methyl-^{14}C]methionine to those derived from [^{14}C]formate and [3-^{14}C]serine should be essentially the same in [methyl-^{14}C]-choline derived from AE and containing three 'new' methyl groups, as in [methyl-^{14}C]-choline derived from MAE and containing two 'new' methyl groups, or [methyl-^{14}C]-choline derived from DMAE and containing only one 'new' methyl group. Inhibition of one of the pathways of S-adenosylmethionine synthesis should decrease the total amount of choline produced in the homogenate. Whether one, two, or three methyl groups are needed to complete the molecule should make no difference, since the various precursors contribute equally to each methyl group. The decrease in choline synthesis through inhibition of one of the synthetic pathways is, therefore, a measure of the contribution of this pathway to the total synthetic process.

With [methyl-^{14}C]methionine as a substrate, inhibition of *de novo* methyl synthesis by Aminopterin eliminates dilution of [^{14}C]choline by nonradioactive choline derived from formate or serine. It thereby decreases the total yield while increasing the specific activity of newly synthesized choline. Fractions containing Aminopterin produce less choline but the same total radioactivity as in control flasks; choline produced in the presence of Aminopterin is of higher specific radioactivity than choline produced in control flasks. This is true for choline containing one, two, or three radioactive methyl groups (Table 2). In our homogenate approximately one-third of each of the three methyl groups of choline arises from exogenous methionine while two-thirds arise by *de novo* synthesis from homocysteine and one-carbon donors.

Since all three groups can, theoretically, arise from methionine only, or from formate only, the ratio of 2 : 1 obtained in our homogenate is purely accidental. With less folic acid it might have been 1 : 1. A harsher treatment which would eiiminate most of the methionine-activating enzyme might produce a 9 : 1 ratio, and so on.

An assumption that all three methyl groups come from S-adenosylmethionine would satisfactorily explain the apparently contradictory findings of DU VIGNEAUD *et al.* (1941) and STEKOL *et al.* (1955) in intact rats. In 1941 DU VIGNEAUD *et al.* stated that "the concentration of deuterium in the methyl groups of choline and creatine from the tissues as well as of the urinary creatinine rose to 85% of that of the deuteriomethionine fed over a period of 14 weeks, strongly indicating that there was no other precursor of methyl groups in the diet employed." Short-term experiments with [$^{14}CD_3$]methionine in which the $^{14}C : D_3$ ratio remained constant eliminated the possibility of transfer of deuterium atoms except as methyl radicals (KELLER *et al.*, 1949). These data indicated that all three methyl groups may be obtained from exogeneous methionine in folic acid-deficeint systems or in animals fed a high-methionine diet which suppressed the endogeneous synthesis of methyl groups. In the determination of the percentage of deuterium in choline only an average value for all 3 methyl groups could be obtained. If any of choline methyl groups were derived from the methyl group of methionine oxidized to the formaldehyde level with the concomitant loss of one deuterium atom, reduction would result in a dilution of the average deuterium content by at least one-ninth. Since the deuterium–protium ratio was the same in creatine, containing one, and in choline, containing three, methyl groups, all four methyl groups must be derived in a similar manner from a common pool.

Folic acid is required for the synthesis of a major portion of choline and the methyl acceptors, MAE and DMAE. According to our data, almost two-thirds of the latter compounds arise by *de novo* synthesis from formate or serine and require folic acid as cofactor. Addition of folic acid to deficient animals should stimulate the synthesis of choline regardless of one-carbon source by providing more MAE and DMAE. Indeed, STEKOL (1958) reported that folic acid stimulated [methyl-^{14}C]choline synthesis from [methyl-^{14}C]methionine. Addition of AE did not overcome the folic acid deficiency. It has been discussed previously (ALEXANDER, 1960*c*) how an excess of AE can decrease the yield of [methyl-^{14}C]choline by pre-empting all available radiomethyl radicals for conversion to MAE, some DMAE, but not choline. According to STEKOL, MAE, unlike AE, enhances cholinesyn thesis. Utilization of [methyl-^{14}C]-methionine for choline synthesis by folic acid-deficient rats receiving a continuous dietary supply of MAE was 26% higher than in control animals and 56% higher than in animals which received a continuous supply of AE (STEKOL, 1958). This implies that MAE can be converted to choline by accepting two methyl groups. DMAE, the immediate precursor of choline, was able to cancel the effect of folic acid deficiency. STEKOL's data with deficient animals showed that there is a progression AE<MAE< DMAE in overcoming the deficiency of folic acid.

Published reports from other laboratories further support our interpretation of the mechanism of biogenesis of the three methyl groups of choline. MELVILLE *et. al.* (1959) showed in the case of ergothioneine that a trimethyl-ammonium moiety can be synthesized by triple transmethylation. CROMWELL and RENNIE (1954) showed that etiolated wheat seedlings form more choline from AE and DMAE with formate than with methionine. NYC (1956) found that [^{14}C] MAE isolated from *N. crassa* is more

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radioactive when formate rather than methionine is used as source of ^{14}C. STEKOL *et al.* (1958) found that a rat liver homogenate which is unable to activate exogeneous methionine can utilize S-adenosylmethionine. MUDD and CANTONI (1958) stated that the mammalian methionine-activating system is more sensitive to inhibitors and more specific in its cofactor requirements than the corresponding yeast system. I have found that it is also more labile during homogenization. The failure of STEKOL's liver homogenate to utilize methionine in choline synthesis proves that the methionine-activating system is more labile than the transmethylating system. Since utilization of [3-^{14}C]serine in our system is inhibited more by S-adenosylmethionine than by free methionine (ALEXANDER, 1960*b*), it is probable that the β-carbon atom of serine can be utilized in choline synthesis without going through free methionine. Whether S-adenosylmethionine is an obligatory intermediate in this process cannot be answered by homogenate experiments alone since many enzymes are known to have been destroyed during homogenization.

The use of free AE and its methylated derivatives in this work does not imply that these compounds rather than their phosphorylated derivatives are the more immediate precursors of choline. BREMER and GREENBERG (1960) isolated [^{14}C]lecithin from rat-liver microsomes incubated with S-adenosyl-[methyl-^{14}C] methionine. WOLF and NYC (1959*b*) showed that the phosphorylated compounds accumulate in a choline-dependent mutant of *Neurospora crassa*. Some form of activation of the aminoethanols is to be expected. Whether or not phosphorylation is the first step in their utilization remains to be determined.

SUMMARY

[Methyl-^{14}C]choline synthesized from aminoethanol and radioactive one-carbon donors in a yeast homogenate is three times as radioactive as [methyl-^{14}C]choline synthesized from dimethylaminoethanol. Aminopterin inhibitssynthes is of choline from serine or formate but does not inhibit synthesis from methionine. Specific activity of [^{14}C]choline synthesized from[met hyl-^{14}C]methionine in the presence of Aminopterin is three times the specific acitvity of [^{14}C]choline synthesized in control flasks containing folic acid. The findings point to the probability that all three methyl groups of choline can be synthesized from methionine by transmethylation in the absence of *de novo* methyl synthesis. In cofactor-rich yeast homogenate approximately one-third of each of the three methyl groups of choline is derived from exogeneous methionine; the remaining two-thirds are synthesized *de novo* from serine and formate probably via adenosylmethionine.

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THE SUBSTRATE SPECIFICITY OF MOUSE BRAIN PEPTIDASE ACTIVITY*

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RECENT studies have revealed the existence of peptides in free form in mammalian brain (UZMAN, 1958*a*) at all ages of post-natal development as well as in invertebrate nerve (DEFFNER and HAFTER, 1959). Studies on human brain have also indicated that some peptides may play a copper-binding role in hepatolenticular degeneration (UZMAN, 1958*b*) and thus bring about the characteristic deposition of copper within glia cells in this disease. It seemed therefore desirable to obtain more information concerning the efficacy and specificity with which peptides with different amino acid compositions and sequences can be split by the mammalian brain. As an initial step towards this end we have undertaken a survey of the peptidase activity of whole mouse brain towards a wide spectrum of peptides representing different structural arrangements of constituent amino acid residues. The choice of mouse cerebral enzyme activity was dictated by the detailed information and experience already available to us on this species both in terms of developmental histology and of chemical composition (FOLCH-PI, 1955; UZMAN and RUMLEY, 1958; UZMAN, 1958*a*). The only information available to us in connection with cerebral peptidase activity has been the earlier meticulous studies of POPE on the enzymic hydrolysis of a single dipeptide, DL-alanyl-glycine, by the different layers of rat (POPE, 1952) and human cortex (POPE, 1959) which indicated the presence of dipeptidase activity in neuronal and glial cell bodies and processes. The present report concerns itself with our observations on the striking quantitative differences in the peptidase activity of mouse brain, under *in vitro* conditions and without addition of co-factors or activators, towards known peptides with different compositions, and is a detailed extension of our earlier brief preliminary report on the N-glycyl–dipeptidase activity of mouse brain (UZMAN *et al.*, 1960).

MATERIALS AND METHODS

The animals used in this study consisted of young adult (9–15 weeks old) Swiss albino mice maintained in the colony at the New Animal House of the Harvard Medical School. For each experiment 2-4 animals were killed by decapitation and exsanguination. The brains were removed within 1 min, rapidly freed of blood and extraneous tissue fluids by touching a clean piece of filter paper, and placed on a glass microscope slide which was in turn placed on a flat slab of dry ice. The whole brain which was thus frozen within a few seconds of removal was trimmed with a razor to yield a standard size block by removing the olfactory poles and the occipital poles at the level of the superior colliculi by two symmetrical frontal sections. The frozen block containing the major portion

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of the two cerebral hemispheres was transferred to a freezing microtome where it was cut into frontal sections 75 μ thick. Sections were immediately transferred with a glass rod into pre-chilled glycerol–phosphate buffer solution, while alternate sections were transferred to adhere in radial fashion to the sides of a glass weighing bottle sitting in an alcohol–dry ice bath. The latter was immediately placed in high-vacuum to yield the total dry weight of alternate sections. The validity of such a step in providing an accurate measurement of the total dry weight of sections placed directly in glycerol–phosphate buffer was repeatedly and periodically tested throughout the course of the study by collecting alternate sections in two separate weighing bottles, freeze-drying both, and weighing them. The weights agreed within 5 per cent of each other.

Freeze-dried sections of known total dry weight were also extracted with the same glycerol–phosphate buffer overnight to yield extracts of dry brain sections. The activity of such extracts was compared with those obtained by direct extraction of frozen wet sections.

The glycerol–phosphate solution used corresponded essentially to that employed by POPE (1959); it consisted of 30% glycerol (Merck) contained in a 4% phosphate buffer (0·067 M, pH 7·0) made up with freshly-boiled and cooled distilled water. In all experiments, 1·0 ml of this solution was used for every 20 mg dry weight equivalent amount of brain tissue slices. The extraction of enzyme was achieved by allowing fresh-frozen or freeze-dried sections to float in this glycerol–phosphate solution overnight (16 hr) at 2° with occasional gentle agitation of the vessel. At the end of this time the extract was centrifuged at 3000 **g** and filtered through a micro sintered-glass filter to yield a clear viscous solution (pH 7·0). This solution was immediately used for incubation with different concentrations of known peptides. Unless specifically designated otherwise, all results refer to extracts directly prepared from fresh-frozen brain tissue-slices.

The incubation and titration were carried out in the same individual micro test-tubes (4 × 25 mm). To 50 μl of extract in glycerol–phosphate buffer (corresponding to about 1·0 mg of dry weight equivalent of fresh slices) 50 μl of peptide solution (ranging in amounts from 1·5 μmoles to 5·0 μmoles) in 0·003 N-NaOH was added. Only with glycyl-DL-aspartic acid and *p*-aminobenzoyl-L-glutamic acid was it necessary to use slightly stronger alkali (0·01 N) to neutralize the peptide solutions of highest concentrations, the lesser concentrations being then obtained by dilution of portions with 0·003 N-NaOH. The tubes were capped, and the solutions were thoroughly mixed by 'buzzing' on the shaft of a high-speed micro-motor. Foaming was diligently avoided at all phases of the experiment. Incubation was carried out for 2 hr in equipment designed to give optimal accuracy of temperature control within the reaction vessels at 34°. For comparison of the temperature-dependence of the total amount of peptide hydrolysed, a separate series was run at 37°. The close agreement between the two series is discussed under Results.

The formol titration was carried out immediately after the incubation. The reaction in each tube was stopped by addition of 50 μl of 37% formaldehyde (Merck, reagent grade) previously neutralized with a few drops of N-NaOH to a light pink with phenolphthalein (pH 8·4–8·6). To each tube 4 μl of 0·1% phenolphthalein in 50% ethanol were added, and each tube was titrated, together with appropriate tissue and peptide blanks, against 0·1 N-NaOH in a micro-titration apparatus similar to that described by LINDERSTRØM-LANG and HOLTER (1935). The peptide and tissue blanks were subtracted from the enzyme plus peptide test values and the results were expressed as μmoles COOH released per mg dry weight equivalent of tissue.

For chromatographic study of the end-product of hydrolysis in each experiment a parallel set of tubes was set up containing the highest concentration of peptide (5·0 μmoles per mg dry weight tissue equivalent) with and without added enzyme extract. These were incubated in identical manner together with each series used for formol-titration. At the end of the incubation 10 μl of the mixture from each tube were spotted on chromatograms, and the splitting, or lack of splitting, of the peptide by the extract was confirmed by paper chromatography. The ninhydrin-positive material in the enzyme extract itself was too low in concentration to interfere, and usually not visible on the chromatograms. The solvent used was butanol–acetic acid–ethanol–water (45:10:35:10) in 16 hr descending runs with drip-off arrangement. The colour was developed with 0·2% ninhydrin in *n*-butanol. For the visualization of proline, the chromatograms were sprayed with 0·2% isatin in acetone to obtain the brilliant blue colour of the proline split from the proline peptides.

For the chromatographic separation of glycine, diglycine, triglycine, and tetraglycine the solvent mixture pyridine–ethanol–water (10:80:10) proved most satisfactory. For the identification of diglycine among the enzymic products when tripeptides containing the diglycine residue were used

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as substrates, paper electrophoresis of the reaction mixture at the end of incubation proved necessary. The Spinco-Durrum electrophoresis cell with 0·33 M-pyridine–acetic acid buffer at pH 4·00 (GRASSMANN *et al.*, 1955) and with runs carried out for 5 hr at a potential gradient of 350 volts gave the best results (Fig. 3).

The peptides used in this study were obtained commercially* and recrystallized or re-purified whenever necessary. Each preparation was tested chromatographically for purity and in some instances by paper electrophoresis, those showing impurities (admixture of free amino acid or other additional ninhydrin-reacting spots) being eliminated from the series. The constituent amino acids of each peptide were checked by chromatography of the acid hydrolysate of about 5 mg of the peptide, the hydrolysis being effected with 6 N-HCl in sealed tubes at 110° for 16 hr.

The higher peptides, carbobenzoxy-L-valyl-S-benzyl-cysteine ethyl ester (P1), L-seryl-L-valyl-L-cysteine (P2), glycyl-L-valyl-S-benzyl-L-cysteine ethyl ester (P3) and glycyl-L-*iso*leucyl-L-valyl-L-α-glutamyl-L-α-glutamyl-L-cysteinyl-L-valyl-L-serine (P4), were synthetic products produced by the di-cyclohexyl-carbodiimide method (SHEEHAN and HESS, 1955). These were also checked before and after acid hydrolysis by paper chromatography for purity and correct composition of constituent amino acids.

RESULTS

Under conditions used to test the enzymic activity of whole mouse brain, it became apparent that an active soluble peptidase could be reproducibly extracted from brain by the procedure used. Inasmuch as pilot experiments had indicated that peptidase activity was not enhanced if the tissue slices were present in the glycerol-phosphate solution at the time the peptide was added, or during the incubation, the use of the clear tissue extract in all the subsequent studies was preferred. In all, 28 experiments with fresh enzyme extracts were performed. In each instance glycyl-L-leucine, which was maximally split at all peptide concentration levels, was included in the particular series of peptides tested as a control for the potency of the enzyme preparation. It made no difference whether the fresh-frozen brain slices were treated directly with the glycerol–phosphate buffer, or were freeze-dried first, and subsequently treated with the same solution. In each instance, the results were identical over the same wide range of peptide concentration (1·5–5·0 μmoles peptide/mg dry weight tissue equivalent enzyme).

The patterns of differential hydrolysis were not affected significantly when incubation at 34° for 2 hr was compared with the efficacy of hydrolysis of individual peptides at 37° for the same period. The following representative peptides: glycyl-L-leucine, glycyl-L-alanine, glycyl-DL-alanine, glycyl-glycine, glycyl-glycyl-glycine, glycyl-γ-aminobutyric acid, L-alanyl-L-leucine, L-leucyl-glycine, glycyl-L-methionine, DL-alanyl-glycine, were incubated in two separate series with the same enzyme preparation at 34° and 37° for 2 hr. The peptides were added to cover the three concentration ranges 1·5, 2·5 and 5·0 moles peptide per mg dry tissue equivalent enzyme in each instance. Titration at the end of incubation showed that all these peptides were hydrolysed to the same extent at 34° as at 37° at the 1·5 μmoles and 2·5 μmoles level. At the 5·0 μmoles peptide level the results were almost the same (within 10%) for all

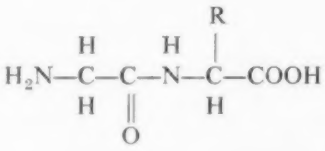
* The sources for the peptides used are indicated below. The numbers designate the number of the peptide in the Tables referred to. California Corporation for Biochemical Research: Table 1, nos. 4, 14, 16 and 24; Table 2, no. 34; also DL-leucyl-glycyl-glycine. General Biochemicals, Inc.: Table 1, nos. 1 and 10; and also triglycine. Mann Research Laboratories, Inc.: Table 1, nos. 3, 8, 9, 13, 17, 22, 23, 25, 27; Table 2, nos. 28 and 29. Nutritional Biochemicals Corporation: Table 1, nos. 2, 5–7, 11, 12, 15, 18–21; Table 2 nos. 30–33; and also L-leucyl-glycyl-glycine and tetraglycine. The higher peptides, carbobenzoxy-L-valyl-S-benzyl-cysteine ethyl ester, L-seryl-L-valyl-L-cysteine, glycyl-L-valyl-S-benzyl-L-cysteine ethyl ester, and the octapeptide glycyl-L-*iso*leucyl-L-valyl-L-α-glutamyl-L-α-glutamyl-L-cysteinyl-L-valyl-L-serine had been prepared by the dicyclohexyl-carbodiimide method and were gifts of H. Rosen, Department of Surgical Metabolism, Walter Reed Army Medical Center, Washington, D.C.

peptides with the exception of DL-alanyl-glycine in which the titrated hydrolysis value was 14% higher at 37° than 34°. Therefore, unless otherwise specified, all results presented here, will refer to experiments in which the incubations were carried out at 34° for 2 hr.

In no instance was there any evidence of utilization of a peptide or its split products either by incorporation into another peptide, or by deamination, although such evidence was diligently sought for by chromatography. The sensitivity of the procedure would have detected any utilization or incorporation above the 10% level of substrate added or end-products released during hydrolysis. In every instance, when a peptide was split, its constituent amino acids were accounted for.

Enzymic hydrolysis of N-*glycyl-dipeptides*

The results of the study of a wide variety of such peptides are presented in Table 1 (nos. 1–21). In many instances two or more separate experiments were performed, a different enzyme solution from the brains of different batches of animals being used in each case. In such experiments the maximal deviation from the mean value is presented in parentheses. Each peptide in this series showed significant differences in the total amount split at high peptide concentration (5·0 μmoles/mg dry tissue equivalent of enzyme). However, apart from these minor differences evident at high peptide concentration, there were major differences in the splitting of these peptides throughout the whole concentration range tested that could be directly related to the structure of the C-terminal amino acid residue.



Thus, when R = H, as in diglycine, the hydrolysis was minimal (Table 1, no. 1). A vast increase in the total amount of peptide split at all concentrations occurred when R represented an aliphatic side chain in an L-amino acid (Table 1, nos. 2–5). When R was represented by an aromatic residue with or without a polar hydrophilic —OH group as in tyrosine and phenylalanine (Table 1, nos. 10, 11) the hydrolysis was still very efficient. Similarly, the substitution of an indole nucleus in the side chain, as in glycyl-L-tryptophan resulted in optimal splitting, even though the poor solubility of this peptide limited the concentration range through which it could be tested. When the N-glycyl-dipeptide had an amino acid residue in the DL-form, only the L-form was split, so that only about half the total amount of peptide was split in conformity with the results obtained when all residues were in the L-form (Table 1, nos. 14–21). The chromatograms in these instances confirmed the presence of intact peptide in addition to the split amino acid constituents. When R was represented by H, but separated by several carbon atoms from the peptide link, as in glycyl-γ-aminobutyric acid (Table 1, no. 6), and consequently possessing no side-chain, the peptide was not hydrolysed at all. This contrasted strikingly with (R = CH_3CH_2–) as in glycyl-DL-α-aminobutyric acid (Table 1, no. 15) in which the L-form of the peptide was wholly split. As with the aromatic residues, the presence of an —OH group on the aliphatic side chain made no

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TABLE 1.—ENZYMIC HYDROLYSIS OF N-GLYCYL AND C-GLYCYL DIPEPTIDES

No.	DIPEPTIDE†		μmoles-COO^- released/mg dry tissue equiv. Peptide added μmoles/mg dry tissue equiv. enzyme			Chromatography of incubation mixture	
			1·5	2·5	5·0	Amino acids released	Peptide
1	Glycyl-glycine	(5)	0·60 (±0·2)	0·66 (±0·2)	0·75 (±0·2)	Glycine	+
2	Glycyl-L-alanine	(2)	1·45 (±0·1)	2·40 (±0·05)	4·47 (±0·05)	Glycine, Alanine	0
3	Glycyl-L-valine	(2)	1·48 (±0·1)	2·32 (±0·1)	4·50 (±0·1)	Glycine, Valine	0
4	Glycyl-L-leucine	(28)	1·50 (±0·1)	2·50 (±0·1)	4·95 (±0·1)	Glycine, Leucine	0
5	Glycyl-L-isoleucine	(2)	1·30 (±0·05)	2·19 (±0·05)	4·32 (±0·05)	Glycine, Isoleucine	0
6	Glycyl-γ-aminobutyric	(4)	0·02 (±0·05)	0·02 (±0·05)	0·02 (±0·05)	0	+
7	Glycyl-L-threonine	(1)	1·36	2·15	4·01	Glycine, Threonine	0
8	Glycyl-L-methionine	(2)	0·17	0·17	0·17	0	+
9	Glycyl-L-asparagine	(1)	1·55	2·40	4·01	Glycine, Asparagine	0
10	Glycyl-L-tyrosine*	(3)	1·50 (±0·05)	2·30 (±0·1)	4·32 (±0·2)	Glycine, Tyrosine	0
11	Glycyl-L-phenylalanine	(2)	1·42 (±0·05)	2·40 (±0·1)	4·82 (±0·1)	Glycine, Phenylalanine	0
12	Glycyl-L-tryptophane**	(1)	—	—	—	Glycine, Tryptophane	0
13	Glycyl-L-proline***	(3)	0·40 (±0·1)	0·40 (±0·1)	0·40 (±0·1)	Glycine, Proline	+
14	Glycyl-DL-alanine	(3)	0·82 (±0·05)	1·34 (±0·1)	2·30 (±0·1)	Glycine, Alanine	+
15	Glycyl-DL-α-aminobutyric	(3)	0·90 (±0·1)	1·45 (±0·1)	2·78 (±0·2)	Glycine, α-aminobutyric	+
16	Glycyl-DL-serine	(1)	0·55	0·94	1·60	Glycine, Serine	+
17	Glycyl-DL-valine	(4)	1·04 (±0·05)	1·60 (±0·05)	2·73 (±0·05)	Glycine, Valine	+
18	Glycyl-DL-leucine	(1)	0·85	1·31	2·42	Glycine, Leucine	+
19	Glycyl-DL-isoleucine	(2)	0·81 (±0·05)	1·22 (±0·1)	2·16 (±0·2)	Glycine, Isoleucine	+
20	Glycyl-DL-phenylalanine	(3)	0·80 (±0·05)	1·26 (±0·05)	2·20 (±0·1)	Glycine, Phenylalanine	+
21	Glycyl-DL-aspartic acid	(1)	0·09	0·35	0·35	0	+
22	L-Alanyl-glycine	(1)	1·52	2·42	4·60	Alanine, Glycine	0
23	L-Methionyl-glycine****	(2)	1·63 (±0·1)	2·50 (±0·1)	4·60 (±0·2)	Methionine, Glycine	0
24	L-Leucyl-glycine	(4)	1·45 (±0·1)	2·20 (±0·25)	2·52 (±0·51)	Leucine, Glycine	+
25	L-Prolyl-glycine***	(3)	0·85 (±0·1)	0·90 (±0·15)	1·0 (±0·15)	Proline, Glycine	+
26	DL-Alanyl-glycine	(4)	0·86 (±0·1)	1·47 (±0·1)	2·43 (±0·2)	Alanine, Glycine	+
27	DL-Leucyl-glycine	(1)	0·79	1·20	1·97	Leucine, Glycine	+

* The tyrosine liberated during hydrolysis at the highest peptide concentration precipitates out during incubation but redissolves in the course of the titration.

** Very poorly soluble, had to be tested with only 0·5 μmoles peptide which was totally split.

*** Free proline gives only 50% of theoretical value by formol titration.

**** Very poorly soluble, had to be tested at levels of 0·75, 1·25 and 2·5 μmoles peptide, values given are read off this curve.

† Figures in parentheses after each peptide represent the number of individual experiments in which the particular peptide was tested.

difference and led to almost total hydrolysis of the L-form of the peptide, as in glycyl-DL-serine and glycyl-L-threonine (Table 1, nos. 16 and 7). The presence of an amide group on the side chain, as in glycyl-L-asparagine (Table 1, no. 9) did not hinder complete splitting. Chromatograms of the incubation products indicated that the end products of the hydrolysis were solely glycine and asparagine. No free aspartic acid was formed. On the other hand the presence of a free carboxyl group on the side chain, as in glycyl-DL-aspartic acid, made the peptide totally immune to hydrolysis under the

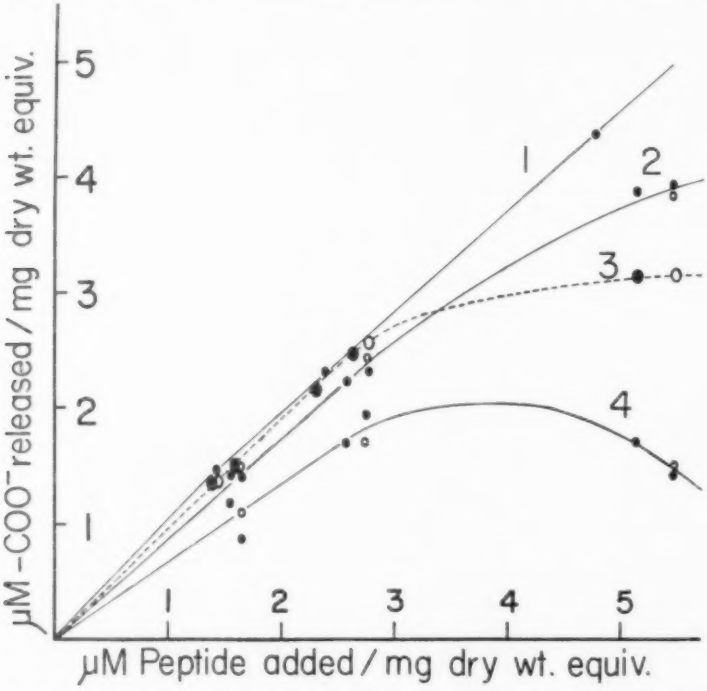

FIG. 1.—Showing the effect of the side chain of the N-terminal amino acid on the hydrolysis of a dipeptide at different peptide concentrations. Curve 1: L-alanyl-glycine; curve 2: L-alanyl-L-leucine; curve 3: L-leucyl-glycine; curve 4: L-leucyl-L-alanine, showing definite inhibition of hydrolysis at high peptide concentrations. Experimental points represented by open circles on curves Nos. 2–4 indicate values obtained with extracts from dry tissue slices in addition to those from the fresh frozen slices.

conditions employed. Glycyl-L-proline was hydrolysed poorly at all concentrations of peptide (Table 1, no. 13). When R was represented by the side chain of glycyl-L-methionine the peptide was also poorly split, but when the position of the same side chain in relation to the peptide bond was different, as in L-methionyl-glycine, the hydrolysis was much more effective (Table 1, nos. 8, 23).

Hydrolysis of C-glycyl-dipeptides

A limited number of these was available for this study as some had to be eliminated from this series when chromatography indicated gross impurity of some of the commercial peptide preparations and attempts at further purification appeared impractical. The nature of the aliphatic side chain of the N-terminal amino acid residue appeared to

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influence very strikingly the amount of peptide split at different peptide concentrations. Thus, glycyl-glycine showed minimal splitting, L-prolyl-glycine, slightly more, L-leucyl-glycine and L-methionyl-glycine decidedly more, while L-alanyl-glycine was maximally split (Table 1, no. 22). The hydrolysis of L-leucyl-glycine was decidedly influenced by variations of the extract in different experiments. Thus, although the peptide was maximally split by all extracts tested at peptide concentrations of 1·5 μmoles/mg dry tissue equivalent of enzyme, the amount split reached a plateau rapidly

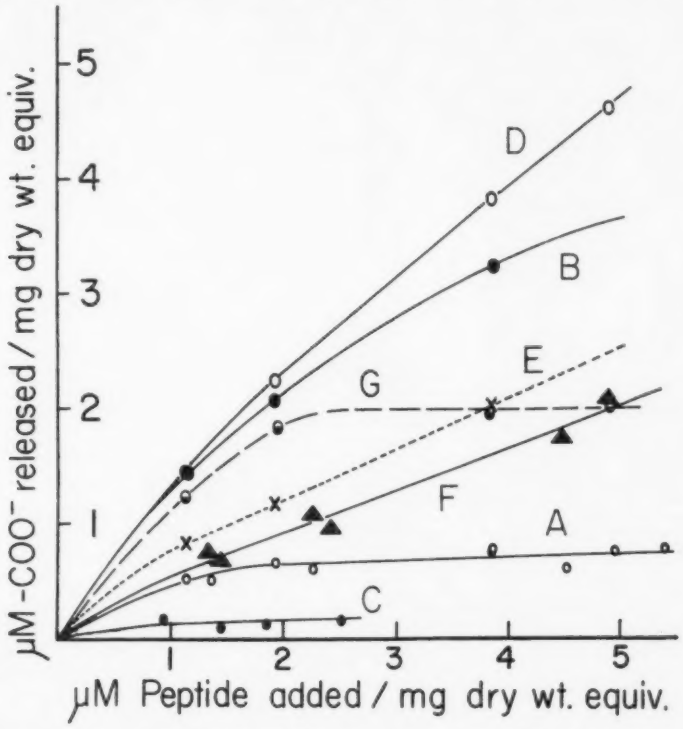

FIG. 2.—Showing the effect of the diglycine residue on the hydrolysis of tripeptides. Curve A: diglycine; curve B: triglycine; curve C: tetraglycine; curve D: L-leucyl-glycyl-glycine; curve E: DL-leucyl-glycyl-glycine; curve F: DL-alanyl-glycyl-glycine; curve G: L-leucyl-glycine.

at higher values, so that in each instance no more was split at 5·0 μmoles/mg dry tissue equivalent than was at 2·5 μmoles/mg dry tissue equivalent. There was a difference, however, in the amount split from preparation to preparation at higher peptide concentrations so that the absolute amount split varied between 2·0–3·1 μmoles at the 5·0 μmoles peptide/mg dry tissue level, (compare L-leucyl-glycine curves in Figs. 1 and 2, and Table 1). In all these instances glycyl-L-leucine, which was used as the control for enzymic activity, was maximally split at all concentration levels.

The true nature of the influence which an aliphatic side chain adjacent to the terminal free amino group of a dipeptide exerted over the pattern of enzymic hydrolysis was, however, not evident until we studied the relationship of this side chain to the side chain adjacent to the terminal carboxyl group (Table 2 and Fig. 1). It became

TABLE 2.—ENZYMIC HYDROLYSIS OF MISCELLANEOUS DIPEPTIDES

No.	DIPEPTIDE†	μmoles-COO^- released/mg dry tissue equiv.			Chromatography of Incubation Mixture	
		Peptide added μmoles/mg dry tissue equiv. enzyme				
		1·5	2·5	5·0	Amino Acids Released	Peptide
28	L-Alanyl-L-leucine (2)	1·40 (±0·1)	2·20 (±0·05)	3·82 (±0·1)	Alanine, Leucine	+
29	L-Leucyl-L-alanine (2)	0·96 (±0·1)	1·65 (±0·1)	1·76 (±0·2)	Leucine, Alanine	+
30	DL-Alanyl-DL-valine (1)	0·19	0·19	0·19	0	+
31	DL-Alanyl-L-leucine (1)	0·29	0·19	0·19	0	+
32	DL-Alanyl-DL-leucine (1)	0·57	0·68	0·95	Alanine, Leucine	+
33	D-Leucyl-L-tyrosine (1)*	0·19	—	—	0	+
34	*p*-Aminobenzoyl-L-glutamic** acid (3)	—	—	—	0	+

* Insoluble at higher concentrations.
** The peptide itself is not amenable to formol titration. Chromatography after incubation with enzyme showed no hydrolysis whatsoever.
† Figures in parentheses after each peptide represent the number of separate experiments from which the data are derived.

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apparent that the introduction of an aliphatic side chain on the C-terminal amino acid residue in some instances could result in a decreased splitting of a dipeptide at high peptide concentrations if the N-terminal amino acid was an aliphatic one other than glycine. Thus, L-alanyl-glycine is split to a greater extent than L-alanyl-L-leucine, while L-leucyl-glycine is hydrolysed to a greater extent than L-leucyl-L-alanine (Fig. 1). The latter in high concentration actually inhibits its own enzymic hydrolysis. This was true with enzyme preparations obtained from freeze-dried brain slices as well as from fresh-frozen ones (Fig. 1). Other dipeptides fitting this scheme were not available for testing and the pattern thus observed could not be established as a general rule in the enzymic hydrolysis of dipeptides by mammalian brain.

Miscellaneous dipeptides

Other than those already mentioned a number of racemic dipeptides of aliphatic amino acids were also tested (Table 2). These were uniformly poorly hydrolysed, if at all. Among others in this group *p*-aminobenzoyl-L-glutamic acid was not hydrolysed at all.

Enzymic hydrolysis of higher peptides

Some of those studied in this category are presented in Fig. 2. Since the glycyl-glycine residue occurs in some of them, the hydrolysis of this dipeptide, included again in the experiments listed, has also been included in Figs. 2 and 3 for comparison. To our surprise, in tripeptides containing the diglycine residue, the hydrolysis of the third amino acid residue proceeded quite independently of the poor hydrolysis of the diglycine residue. Thus, triglycine on hydrolysis yielded readily glycine and diglycine, the latter showing some independent minimal splitting (Fig. 2). The information obtained by formol titration alone was ambiguous until the identity of the end products was unequivocally established by paper electrophoresis (Fig. 3). Similarly, L-leucyl-glycyl-glycine showed optimal splitting of leucine to yield leucine and diglycine as the end-products of hydrolysis at all concentration levels tested (Figs. 2 and 3). This stood in marked contrast to the relatively poor hydrolysis of L-leucyl-glycine when present as a dipeptide (Table 1, Fig. 2). Again, DL-alanyl-glycyl-glycine liberated alanine and diglycine. In striking contrast to the hydrolysis of triglycine, tetraglycine was not hydrolysed at all (Fig. 2) within the concentration ranges its low solubility permitted testing. Thus, the diglycine residue in a peptide was treated by the enzymic system as an independent unit, the N-terminal amino acid attached to this unit bearing no relationship in the pattern of its hydrolysis from this unit to the pattern observed when this same amino acid occurred as the N-terminal residue of a C-glycine dipeptide.

Studies on the hydrolysis of other available higher peptides indicated that if a tripeptide possessed a dicarboxylic acid such as glutamic acid as its N-terminal residue or a hydroxy amino acid such as serine, then the hydrolysis was completely inhibited. Thus glutathione, tested in the form of two different commercial preparations (Schwarz, and Nutritional Biochemicals Corporation, the latter re-purified) showed no evidence of splitting by chromatography, nor did L-seryl-L-valyl-L-cysteine. Blocking the free carboxyl group of a higher peptide (glycyl-L-valyl-S-benzyl-L-cysteine ethyl ester) by forming the ethyl ester produced no interference in its splitting. The end products of hydrolysis were identified chromatographically as consisting of glycine,

valine, S-benzyl-cysteine ethyl ester and some unsplit original peptide. Blocking of the free amino group as in carbobenzoxy-L-valyl-S-benzyl-cysteine ethyl ester resulted in no hydrolysis, while substitution of a glycyl residue for the carbobenzoxy residue (as in glycyl-L-valyl-S-benzyl-L-cysteine ethyl ester) resulted in hydrolysis. The former, owing to its total lack of solubility, had to be used as a fine suspension in the glycerol-phosphate solution. Hence, this result is open to question. Perhaps of greater interest was the lack of splitting observed when a highly water-soluble octapeptide, glycyl-L-isoleucyl-L-valyl-L-α-glutamyl-L-α-glutamyl-L-cysteinyl-L-valyl-L-serine, was incubated with brain peptidase. The clear solution formed on mixing this peptide with the enzyme at the beginning of incubation turned into an opaque thixotropic gel at the end of incubation. The total lack of splitting in this case, confirmed chromatographically in three separate experiments, contrasted strikingly with the total hydrolysis observed when glycyl-L-isoleucine was presented to the same enzyme in dipeptide form (Table 1, no. 5).

DISCUSSION

As with most studies of enzymic activity, the observations made in this study and the differences recorded must be considered valid only within the context of the experimental conditions that have been defined in the testing of such activity. The present study reveals that there are rigid structural requirements that a dipeptide has to meet before it can become susceptible to a particular type of hydrolysis by cerebral tissue. These requirements have been defined in detail for N-glycyl dipeptides, and tripeptides containing diglycine residues, while the definition has been less comprehensive for C-terminal glycine dipeptides and other di- and tri-peptides. That the pattern of structural requirements for the hydrolysis of N-glycyl dipeptides is also rigidly maintained for dipeptides like glycyl-γ-aminobutyric acid and glycyl-α-aminobutyric acid, which are not known to occur as natural constituents of any protein, we consider to be of special significance as indicative of a general characteristic of the cerebral dipeptidase valid beyond the limits of our particular experimental conditions. The present study offers no clues as to whether one or many separate enzymes are involved in the specific activities recorded for this particular type of substrate. Nor do we know what co-factors or side reactions would normally be necessary to endow cerebral peptidase activity with greater versatility. These points will have to be dealt with in future studies. It is, however clear that the enzymic system studied makes a distinction between the hydrolysis of a peptide bond when it occurs in a dipeptide and the same bond when it occurs in a tripeptide as exemplified by the difference in the hydrolysis of leucyl-glycine and leucyl-glycyl-glycine (Fig. 2), diglycine and triglycine (Figs. 2 and 3), as well as distinguishing a dipeptide from a higher peptide as observed in the case of glycyl-isoleucine and the octapeptide (P4) containing the same residue in an accessible N-terminal position (Table 1). It therefore appears that the enzymic system studied here defines its substrate-specificity not only in terms of the structural configurations prevailing immediately adjacent to a given peptide bond, but also in terms of other side chains at a distance from that particular peptide bond. This we interpret as indicative of steric specificity of the enzyme system for peptides of different chain lengths. Consequently, doubts must be raised as to whether cerebral peptidase activity studied by the hydrolytic activity towards any short-chain peptide can be representative of any natural proteolytic activity associated with protein turnover in any part of the brain.

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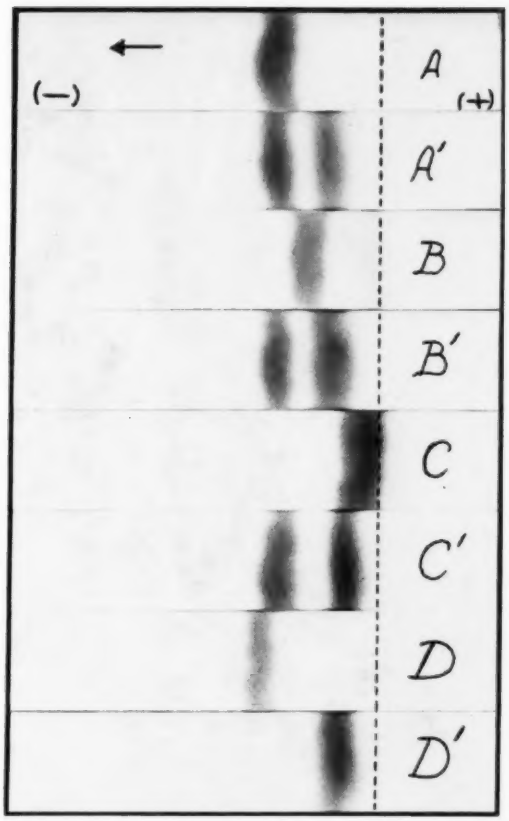

FIG. 3.—Electrophorograms showing the resistance of the diglycine residue to hydrolysis and how it affects the hydrolysis of tripeptides. Run in pyridine–acetic acid buffer (pH 4·0) for 5 hr at 350 volts, stained with ninhydrin 0·2 % in butanol. Dotted line indicates point of application of samples.

A: Glycyl-glycine incubated in glycerol–phosphate solution.
A′: Glycyl-glycine incubated with peptidase, small amount of glycine released, bulk of diglycine still intact.
B: Glycyl-glycyl-glycine incubated in glycerol-phosphate solution.
B′: Glycyl-glycyl-glycine incubated with peptidase, complete splitting into glycine plus diglycine.
C: L-Leucyl-glycyl-glycine incubated in glycerol-phosphate solution.
C′: L-Leucyl-glycyl-glycine incubated with peptidase, splitting into diglycine and leucine.
D: Glycyl-L-leucine incubated in glycerol-phosphate solution.
D′: Glycyl-L-leucine incubated with peptidase, completely split into glycine and leucine which run together in over-lapping slow bands.

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Evidence to be presented in detail in a subsequent publication indicates that the peptidase system (consisting of one or more enzymes) studied here requires no addition of cobalt, manganese, or zinc to activate enzymic action. No activators were added in this study. In this respect this enzymic system differs from the peptidases of serum (FLEISCHER, 1953, 1954) which require cobalt or manganese as activators, or the soluble peptidase system reported in leucocytes (FLEISCHER, 1956) which is wholly dependent on the addition of zinc for activation. On the other hand, the enzymic system studied by us does bear a superficial resemblance to the calf thymus tripeptidase described by ELLIS and FRUTON (1951) in the similarity in its splitting of tripeptides containing a diglycine residue, the latter remaining intact during hydrolysis. The brain peptidase differs from the calf thymus tripeptidase in that the latter shows no activity towards dipeptides, especially glycyl-L-proline, and also in that its activity is optimal at pH 8·0. Further differences will be documented in a subsequent communication dealing with the properties of the brain peptidase activity itself.

From a methodological standpoint it would appear that glycyl-L-leucine constitutes the ideal substrate for assay of general dipeptidase activity as long as the conditions used are similar to ours. The substrate specificities recorded indicate that such a choice may have to be modified whenever enzymic activity is tested under significantly different conditions. Only in the cases of L-leucyl-glycine and L-leucyl-L-alanine have we been able to show an inhibitory effect of high peptide concentration. This does not rule out the possibility that the other peptides may exhibit such inhibition if still higher peptide concentrations had been used. This possibility gains special prominence in the interpretation of ultra-micro enzymic studies where a wider difference in the enzyme: substrate ratio may have to be used to permit accurate quantitation of hydrolysis.

SUMMARY

Mouse brain peptidase activity was studied, over a broad range of peptide concentration, towards 34 dipeptides and 10 higher peptides with the object of determining the structural requirements a substrate must meet for enzymic hydrolysis to occur. Under the conditions employed, the following results were obtained:

(1) A lipophilic side chain, aliphatic or aromatic, on the C-terminal amino acid of a N-glycyl dipeptide is essential for hydrolysis to occur. Hydrolysis is inhibited if this side chain becomes hydrophilic, as when replaced by the side chain of a dicarboxylic amino acid, but not when replaced by the amide of the dicarboxylic amino acid.

(2) The presence of an aliphatic side chain on the N-terminal amino acid of a dipeptide in some cases decreases, and at high peptide concentration inhibits the amount of peptide hydrolysed if the C-terminal amino acid also possesses an aliphatic side chain.

(3) The hydrolysis of a given peptide bond is influenced not only by the character of the side chains immediately adjacent to it, but also by the nature of side chains at a distance from it, as demonstrated by the enzymic distinction made between the hydrolysis of a N-terminal amino acid residue occurring in a dipeptide and the same amino acid occurring in the same position in a higher peptide.

(4) The presence of D-amino acid residues in most racemic peptides studied did not affect the hydrolysis of the L-forms if the substrate was presented as a racemic peptide.

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THE UPTAKE OF A METABOLICALLY INERT AMINO ACID BY BRAIN AND OTHER ORGANS*

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A NUMBER of studies have shown that the blood–brain barrier serves to keep the amino acid content of brain relatively constant. FRIEDBERG and GREENBERG (1947) demonstrated that the amino acid level of brain, unlike that of kidney and liver, is largely unresponsive to increases in plasma amino acids. More recently, LAJTHA (1958) reported a similar finding with regard to lysine uptake by adult mouse brain. Evidence also exists that increased blood concentrations of glutamic acid (SCHWERIN, *et al.*, 1950; LAJTHA, *et al.*, 1959) and proline (DINGMAN and SPORN, 1959) are not reflected by upward changes in the brain amino acid pool. The fact that the net uptake of amino acids cannot be forced by increasing plasma levels does not preclude the possibility of rapid exchanges between blood and brain amino acids. LAJTHA *et al.* (1957) and LAJTHA (1959) reported that labelled lysine and leucine reach concentrations in brain equal to serum levels within minutes after injection into the circulation. A recent study by CHIRIGOS *et al.* (1960) with radioactive tyrosine also showed rapid entry in brain, with net uptake after massive doses.

The research described in this report is part of a project devoted to assessing the biosynthetic function and transport activities of the brain. Since it cannot be presumed that the rate of passage of metabolites across the blood–brain barrier is not influenced by the demands of the brain's biosynthetic activities, it was deemed necessary to carry out these studies with a model substance which entered the brain by a normal route, but which was not metabolized or incorporated into protein. Evidence has been accumulated that α-amino*iso*butyric acid, an unnatural amino acid, is transported into cells by the same mechanism responsible for the uptake of normal amino acids (CHRISTENSEN and RIGGS, 1956). The inert character of this model amino acid was exploited in a number of other studies (NOALL *et al.*, 1957; RIGGS and WALKER, 1958) and seemed ideally suited for the purpose of determining blood–brain barrier function. The uptake of this unnatural amino acid in the absence of any degradative or synthetic pathway for it in brain, is perhaps a more valid measure of the capacity of the blood–brain barrier to transport amino acid-like substrates since the kinetics of the process are unmodified by any metabolic activity in the brain.

METHODS AND MATERIALS

Experimental. Unanaesthetized female rats (180–207 g) fasted for at least 16 hr were injected either intraperitoneally or in a tail vein with radioactive α-amino*iso*butyric acid. In experiments of 20 hr duration or longer, the fasting period began after injection. The [1-^{14}C] α-amino*iso*butyric acid (California Foundation for Biochemical Research) contained approximately 8 μc of activity per mg.

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A dose of 8·0–8·4 μC in 0·8 ml of isotonic saline was used. The animals were decapitated at fixed intervals, the blood being collected in ethylenediamine tetraacetic acid-coated tubes, and the plasma was separated by centrifugation. The organs were rapidly excised, rinsed in cold saline, blotted dry, and weighed. Homogenization was carried out in distilled water. Protein was removed from liver and kidney homogenates by heating in a boiling water bath for 8 min after which sufficient trichloroacetic acid (TCA) was added to raise the concentration to 0·5%. TCA was added to the brain homogenate to make a final concentration of 5% to remove protein. One wash after centrifugation left less than 3 per cent of the radioactivity in the protein precipitate. The TCA was extracted with ether. Plasma protein was removed by the addition of 10 vol of 95% alcohol. Portions were evaporated in digestion flasks for counting.

The experiments on young rats were performed on 2–4 day-old animals taken from several litters. The amino acid was injected intraperitoneally in volumes of 0·08 ml or less. Specific activity of the α-amino*iso*butyric acid was 48·5 μC/mg. The amount of material injected per gram body weight (4·3 μg/g) was about 20 per cent less than the amount injected into adults (5·2 μg/g). The tissues were homogenized in distilled water, and portions dried for determination of radioactivity. Blood was weighed on aluminum foil, dried, and transfered to combustion flasks.

In some experiments with adult animals, it was observed that intraperitoneal injections gave higher tissue radioactivities after 10 minutes than intravenous injections into the tail. LAJTHA (1958) reported data which also showed higher tissue values of lysine after intraperitoneal injection. Some variation was also found in tissue levels of α-amino*iso*butyric acid with different injection routes at the longer time intervals. Since the pattern of uptake was the same for both routes, the data were pooled. The results with the animals killed after 10 minutes are omitted because of the uncertainty as to the rapidity with which α-amino*iso*butyric acid distributes after tail vein injection. This early point was not needed to locate the uptake maxima.

Determinations of radioactivity. A model 6000 Dynacon Electrometer (Nuclear-Chicago) was used for measuring radioactivity. The samples were combusted to CO_2 in a sealed glass system and flushed into evacuated ion chambers by a stream of compressed CO_2. With this apparatus radioactivity is directly proportional to amperage as indicated by a meter reading. Since α-amino*iso*butyric acid is not a constituent of the body, the measured radioactivity is directly proportional to the amount of α-amino*iso*butyric acid in the tissues. One μC with this apparatus gave a current of $4{\cdot}6 \times 10^{-12}$ A; no corrections for background current were necessary with samples as small as one-thousandth of a μC.

*Metabolism of α-amino*isobutyric acid. Several experiments were carried out to confirm the metabolic inertness of α-amino*iso*butyric acid in brain. Forty-three μC of α-amino*iso*butyric acid (specific activity 48·5 μC/mg) were injected into a 200 g female rat which was executed at the time of maximal labelling in the brain (20 hr). The brain was homogenized in distilled water and brought to a volume of 25 ml. The radioactivity of the homogenate was determined. Protein was precipitated from another portion, washed several times with dilute TCA, dried in an oven, and combusted to CO_2. No radioactivity could be detected in the protein precipitate. Two ml of the brain homogenate contained approximately 0·029 μC of radioactivity. A detectable change in the background current of the instrument would have occurred if 0·2% of this amount of radioactivity had been incorporated into brain protein. Experiments of 4 hr duration with lower levels of radioactivity also showed no incorporation into protein.

Seven ml of the above homogenate were treated with TCA and the supernatant fluid, after removal of the TCA, was placed on a Dowex 50 (H form) resin column. The material passing through the column (carbohydrates, neutral compounds) was collected, dried, and combusted. No radioactivity was present in this wash. After eluting with 2 N-ammonia and drying the eluate, a portion of the amino acid fraction was chromatographed on paper with an unaged butanol–acetic acid–water (5:1:5) system. The paper strips were scanned with a Geiger-Muller detector, and a single peak was found with the same R_F (0·45) as that of a control spot of α-amino*iso*butyric acid. The sensitivity of the scanning was sufficient to have detected another peak containing only 3 per cent of the radioactivity of the main spot. These experiments indicate that α-amino*iso*butyric acid is chemically unchanged in the brain.

To find if the brain could decarboxylate the labelled carboxylic acid group of α-amino*iso*butyric acid, a 10% rat-brain homogenate was prepared in phosphate buffer (pH 6·8, final concentration 0·025 M). A total of 1·2 mg of pyridoxal phosphate, 51 mg of α-amino*iso*butyric acid plus 0·6 mg of labelled α-amino*iso*butyric acid (4 μC) was added to 16 ml of homogenate. Samples were withdrawn

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at zero time. After 2 hr of incubation at 35°, portions were taken for radioactivity determinations. The samples were first acidified to drive off any CO_2 released into the solution. No decarboxylation of α-amino*iso*butyric acid occurred under these conditions.

Blood determinations. A convenient assay of the amount of plasma in brain was necessary in order to determine whether or not plasma contributed significantly to the radioactivity of brain samples. The method used involved homogenizing brain in isotonic sucrose and centrifuging down the red cells at 1000 **g**. The red cells were homogenized in 2 N-HCl and after a 2 hr waiting period the acid hematin which formed was extracted into an ether layer with vigorous mechanical stirring. The ether layer was drawn off after centrifugation and read in a Beckman DU spectrophotometer at 380 mμ in stoppered cuvettes. The standard for comparison was prepared from 0·1 ml of blood from the same animal. The blood was diluted to 50 ml with 0·1 N-HCl, and 1 ml of this solution was extracted with ether and read simultaneously with the brain red cell acid hematin solution. Appropriate calculations allowed a fairly accurate estimate (within 10 per cent) of the blood contained in a weighted amount of brain. Approximately 5 per cent of brain radioactivity could be attributed to plasma α-amino*iso*butyric acid. This correction became less important as brain label increased and plasma label decreased in experiments of longer duration. Since plasma radioactivity in the brain rarely exceeded 5 per cent, the acid hematin method was not routinely used, and the data presented are not corrected. Liver and kidney values for radioactivity were not corrected for plasma radioactivity since the uptake data were not significantly changed by errors of a few per cent.

The α-amino*iso*butyric acid in the brain sucrose supernatant solutions was recovered by TCA deproteinization, ether extraction of the TCA, and separation of the sucrose from the label on Dowex 50 columns. The amino acid was combusted to determine brain radioactivity. The removal of the brain cell nuclear fraction with the red cells did not affect the results since little radioactivity entered the nuclei.

RESULTS

The results (Table 1) show that the brain differs in its uptake of α-amino*iso*butyric acid in two respects from liver and kidney. First, peak radioactivity is reached by liver

TABLE 1.—α-AMINOISOBUTYRIC ACID IN RAT ORGANS

Time after injection	Number of rats	Brain	Liver	Kidney	Plasma	Brain / Plasma	Liver / Plasma	Kidney / Plasma
			(μg/g tissue)					
20 min	7	0·38	7·9	67·9	6·91	0·055	1·15	9·8
40 min	8	0·54	11·9	94·8	5·88	0·093	2·02	16·1
80 min	7	0·90	14·1	134·0	4·08	0·220	3·44	32·8
4 hr	12	1·93	11·8	119·9	2·75	0·702	4·27	43·6
12 hr	3	3·59	8·9	55·1	2·26	1·59	3·92	24·4
20 hr	4	4·49	7·0	63·1	2·18	2·06	3·21	28·9
30 hr	3	3·48	6·4	46·3	1·50	2·32	4·26	30·9
40 hr	1	2·94	3·6	29·4	1·28	2·30	2·81	23·0

and kidney at about 80 minutes whereas the brain continues to accumulate label for about 20 hours. Second, the amount of radioactivity in brain is always considerably less than in liver. At about 20 hours, when liver has been losing radioactivity for over 16 hours and when brain is at its maximal value, liver still contains 60 per cent more α-amino*iso*butyric acid per gram of tissue. Besides its slower and lesser uptake of label, the brain also loses radioactivity slower than liver.

Kidney radioactivity on a gram wet weight basis ranged from about 6 to 12 times that of liver. Kidney/plasma, liver/plasma, and brain/plasma radioactivity ratios (Table 1) for the longer time intervals are close to those obtained by NOALL *et al.*, (1957) and RIGGS and WALKER (1958) in animals after 30 and 39 hours despite the fact

that the present data are based on radioactivity per gram organ wet weight and not on radioactivity of the intracellular fluid. Table 1 also shows the slow decline of plasma radioactivity over the time interval of the study. The brain continues to accumulate label even while plasma levels are slowly falling. The fact that all three organs ultimately contained more radioactivity per gram than was found in 1 ml of plasma indicates that the model amino acid was actively transported into the tissues.

Some studies were made also on the red cell content of label. Red cell/plasma ratios were about 0·4 after 10–20 minutes, 0·8 after 80 minutes–4 hours, and 0·6 after 20 hours. The ratio after 12 hours was slightly larger than 1·0. Mammalian erythrocytes are known to have little ability to concentrate amino acids (CHRISTENSEN *et al.*, 1952*b*).

Several experiments were devoted to comparing label in cortex and sub-cortex. The protocol and results of a typical experiment were as follows: 3 hr after injection of 18 μC of α-amino*iso*butyric acid, the brain was removed and chilled for 5 min in a deep freezer. The cerebellum and brain stem were discarded. The cortex of the cerebrum was carefully cut away with a razor. On a gram basis, the cortex contained 0·041 μC of activity; the sub-cortical mass contained 0·027 μC.

In another experiment, 10·3 μC of label were injected, and the animal was sacrificed after 165 min. The nuclei and mitochondria from liver and brain were isolated by centrifugation from a 0·25 M-sucrose homogenate, and the radioactivity was determined. These particulate fractions contained only a few per cent of the label present in the original homogenates. These small amounts of radioactivity can be attributed to contamination of the particulate fractions by the much more highly radioactive supernates. A duplicate experiment gave essentially the same results.

In view of the observations of LAJTHA (1958) that the uptake of lysine was higher in new-born and young mouse brain than in adult brain, some experiments were performed on immature rats. In comparing the data (Table 2) with those from the

TABLE 2.—α-AMINOISOBUTYRIC ACID IN ORGANS OF YOUNG RATS

Time after injection (min)	Number of animals	Brain	Liver	Blood	Brain / Blood	Liver / Blood	Brain / Liver
			(μg/g tissue)				
40	5	0·84	2·7	1·6	0·53	1·7	0·31
80	5	1·00	2·9	1·1	0·91	2·6	0·35

adult rats (Table 1), it should be noted that for young rats organ/blood and not organ/plasma ratios are listed. Organ/blood ratios for young animals were identical to organ/plasma ratios since three experiments on 6 day-old rats showed equal volumes of red cells and plasma to have approximately the same content of radioactivity. Hematocrits averaged 0·35 in accord with values in the literature. It is apparent that the brain/blood ratio for young animals is much higher than for adults; this indicates a larger uptake of α-amino*iso*butyric acid. The brain/blood ratio for young rats at 40 minutes is 0·53 and at 80 minutes is 0·91. These values are very considerably higher than for adult animals.

The brain/liver ratios at 40 minutes and 80 minutes are 0·31 and 0·35. For adult animals at 40 and 80 minutes, the ratios are 0·046 and 0·065. These differences do not

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appear to be due exclusively to the greater uptake of label by brain. Since the liver/blood ratios for the young rats are smaller than for adults, it is evident that the livers of young rats are less active in accumulating α-amino*iso*butyric acid.

Two kidneys from young animals at each time interval were also worked up for radioactivity. At 40 minutes the kidney/blood and brain/kidney ratios for these two animals were 7·0 and 0·08 and at 80 minutes were 7·4 and 0·12. Comparison with adult kidney/plasma ratios of 16 and 33 shows that the immature animals have less active kidneys insofar as accumulation of α-amino*iso*butyric acid is concerned. For adult rats, the brain/kidney ratio was approximately 0·006. Since the data do not support the supposition that young brains have an uptake 10 or more times higher than adult brains, it is apparent that the kidneys of young rats have a diminished ability to concentrate label, as is the case also for young livers.

An absolute comparison shows slightly more α-amino*iso*butyric acid in young rat brains than in adult brains. This resulted despite the use of about 20 per cent less amino acid per g body weight in the experiments with young animals. Also, the circulating blood of the infant rats had only one-third the α-amino*iso*butyric acid content of adult plasma. The livers of the immature animals contained about one-fifth as much α-amino*iso*butyric acid on a gram basis as did adult livers.

The infant rats were not fasted as were the adults but this factor was not responsible for the differences observed. A number of experiments on fed adult rats gave tissue radioactivities similar to fasted animals.

DISCUSSION

The smaller and slower uptake of α-amino*iso*butyric acid by brain than by kidney and liver is best explained by invoking the operation of a barrier phenomenon. The slow expulsion of the model amino acid is also in accord with this interpretation and suggests that the barrier functions to retard both uptake and release of label by brain. If the data in Table 1 are plotted on a graph, it can be seen that brain radioactivity equals plasma radioactivity after about 6 hours. For adult mice, free radioactive lysine (LAJTHA *et al.*, 1957) and radioactive leucine (LAJTHA, 1959) in brain have specific activities equal to those of free plasma lysine and leucine in under 20 minutes. This time interval also approximates the peak specific activities of free lysine and leucine in the brain. Recent work with labelled tryosine (CHIRIGOS *et al.*, 1960) showed maximal brain uptake in about 30 minutes and brain radioactivity equal to plasma radioactivity in about 60 minutes. In contrast, maximal uptake of the model amino acid occurs about 20 hours after injection. LAJTHA's calculations indicate that free lysine in brain is replaced by plasma lysine in about one hour. Since LAJTHA reports 37 μg of free lysine per gram of mouse brain, and this quantity turns over in 1 hour, the rate of exchange is seen to be quite considerable when compared to the uptake of model amino acid. After 20 hours, the calculated amount of α-amino*iso*butyric acid accumulated per gram of rat brain is only 4·5 μg, a quantity that represents about 0·2 per cent of the total free α-amino-nitrogen in this organ. This low uptake occurs despite the fact that the quantity injected raised the plasma α-amino-nitrogen an estimated 10 per cent.

The rapid exchange between brain and plasma lysine and leucine and the slow uptake of α-amino*iso*butyric acid is the chief point of interest emerging from these experiments. The factor which might be responsible for the slow accumulation of α-amino*iso*butyric acid is the metabolic inertness of this substance. It is reasonable to

assume that protein incorporation and other biosynthetic or degradative pathways for normal amino acids create a constant demand for substrate which encourages the passage of additional molecules across the blood–brain barrier. Since α-amino*iso*-butyric acid does not undergo metabolic transformations, no stimulus exists to encourage the entry of replacement molecules into the brain. If this is so, LAJTHA's experiments give an indication of the ability of the blood–brain barrier to transport substrates under the stimulus of intense metabolic activity while the results obtained with the unnatural amino acid are a measure of the function of the barrier when no metabolic influences are operative.

Another factor worth considering is the possibility that the model amino acid is stereochemically too different from normal amino acids to be readily transported by the brain's presumably highly specific transport system. This possibility cannot be eliminated without further investigation though it should be mentioned for one type of cell at least, mouse ascites carcinoma, α-amino*iso*butyric acid accumulates more readily than either leucine or lysine (CHRISTENSEN *et al.*, 1952*a*).

Though the expulsion of the model was not studied over a long time interval, it is clear that 10 hours after the peak accumulation at 20 hours the radioactivity of the brain had declined only 20 per cent. Since it was demonstrated that there was no significant radioactivity in nuclei and mitochondria almost 3 hours after injection, the exchange of brain α-amino*iso*butyric acid with the plasma was theoretically uncomplicated by uptake into sub-cellular particulate matter. Nevertheless, the blood–brain barrier seemed to prevent the escape of the compound quite as strongly as it resisted its original entry.

The finding that the cerebral cortex contains more label than the sub-cortical mass is in accord with similar experiments on inorganic [^{32}P]-phosphate and [^{35}S]-methionine (BAKAY, 1956; FLANIGAN *et al.*, 1957).

The results obtained with the immature rats support LAJTHA's (1958) finding that the blood–brain barrier is less effective in young animals. A significant observation in the present study was the very diminished uptake of α-amino*iso*butyric acid by the livers and kidneys of young rats. NOALL *et al.* (1957) reported a greater amino acid 'hunger' in the tissues of younger and smaller rats than of adults, and this was correlated with lower plasma levels of radioactive α-amino*iso*butyric acid. While the data in Table 2 show less amino acid in blood of young rats, in agreement with NOALL, the immature rats in this study did not show a proportionate increase in liver and kidney levels of α-amino*iso*butyric acid. It is probable that NOALL's observations apply to rats considerably older than the ones used in this study.

The blood–brain barrier is one of the brain's distinct physiological attributes and possesses great importance in any theory which attempts to correlate the biochemical functions of the brain as a whole. The ability to deal with the brain's transport mechanism as an independent system by the use of an unmetabolized amino acid offers many advantages by eliminating variables which might otherwise obscure uptake studies. Should future investigation demonstrate that the differences between natural amino acid and α-amino*iso*butyric acid uptakes are a function of the greater specificity of the transport system of the brain for the former, then this fact in itself would deepen our understanding of the mechanisms involved in blood–brain transport phenomena.

The reference data provided are being used in experiments now in progress to

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determine the influence of hormones and drugs on blood–brain barrier function with regard to amino acid like substrates.

SUMMARY

Data have been presented on the uptake of radioactive α-amino*iso*butyric acid, a metabolically inert amino acid, by rat brain, kidney, and liver over a 30-hour period. The differences between the uptake of α-amino*iso*butryic acid and some naturally occurring amino acids were discussed with a view toward elucidating blood–brain barrier function. Studies on immature rats confirmed the lesser effectiveness of the blood–brain barrier for amino acids in young animals.

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DETERMINATION OF TOTAL LIPID IN THE CEREBROSPINAL FLUID BY PAPER CHROMATOGRAPHY

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THE isolation, fractionation, detection, and determination of the lipid components of cerebrospinal fluid (CSF) pose a difficult problem. The volume of CSF normally available for study is small (usually 8 to 10 ml) and its total lipid content is of the order of from 1·0 to 2·0 mg per 100 ml. Lipids not only occur in trace amounts in CSF, but also have been reported to be a complex mixture of widely different chemical types (TOURTELLOTTE, DE JONG and VAN HOUTEN, 1958; TOURTELLOTTE, 1959; GREEN, 1958). The separation of the individual members of this mixture is complicated by such factors as their (1) intersolubility, (2) susceptibility to intramolecular rearrangements, enzymic action, and hydrolytic or oxidative changes, and (3) the possibility of complexing with proteins and other CSF components.

These difficulties have seriously hampered the development of suitable procedures for the routine analysis of lipids in CSF (TOURTELLOTTE, VANDER, SKERNTNY and DE JONG, 1958; SELBACH and TRAPPE, 1944). Hence, it has been impossible to explore adequately the possibility that certain degenerative changes in the central nervous system may be mirrored in concommitant alterations of the lipid pattern in CSF. The present study represents an attempt to facilitate the attainment of this end by adapting suitable chromatographic techniques to the scale of observation demanded by the low lipid content of CSF.

MATERIALS AND METHODS

Fluid samples. Pooled CSF samples were obtained from patients undergoing pneumoencephalograms who had no detectible degeneration in the central nervous system. The CSF was collected under conditions which insured freedom from harmful contamination, transported from the ward to the laboratory in an ice chest, immediately centrifuged at about 150,000 g in a Preparative J Rotor run in a Spinco Model E Ultra-centrifuge for 10 min, and stored in a deep freeze until needed. Two lumbar CSF samples, treated in an identical manner, were also used in the study. These were obtained from individual patients diagnosed to have diffuse (AUSTIN, 1957) and multiple sclerosis.

Reagents. All chromatographic and extraction solvents were reagent-grade chemicals that were redistilled prior to use. The reagents required to carry out the total lipid procedure developed by TOURTELLOTTE and his co-workers were purified and prepared as prescribed by these investigators (TOURTELLOTTE *et al.*, 1958*b*). Water used in preparing the CSF 'blanks', constantly used throughout the study for evaluation purposes, was obtained by treating ordinary distilled water for several hours with $KMnO_4$ acidified with H_2SO_4 and distilling in an all glass apparatus. The distillate was treated with $Ba(OH)_2$, redistilled, and passed through a column of Amberlite MB-1 mixed bed resin. Plasma samples were assayed for total lipid content by SPERRY's gravimetric procedure (SPERRY and BRAND, 1955) and used as standards in the total lipid determinations.

Lipid extraction. Lipid extracts were prepared by a procedure which on the basis of careful preliminary experimentation appeared to be the one of choice. This procedure was carried out by

(1) adding anhydrous methanol–methylal (1:4 v/v) and a small quantity of incinerated sand directly to a portion of CSF, (2) allowing the mixture to stand 30 min at 0° and centrifuging, and (3) removing the supernatant fluid and evaporating to dryness in a rotary evaporator under vacuum at 37° (DELSAL, 1944). The residues were redissolved in petroleum ether (b.p. 60°–70°) or benzene–amyl alcohol (1:1 v/v). The initial volume of CSF employed in applying this and all other extraction procedures was usually 8 to 10 ml.

Total lipid determinations. These were carried out on petroleum ether re-extracts of CSF lipids by applying the latter in the form of a band to strips of fat-free Whatman #1 or Schleicher and Schuell #2042 filter paper, 30 cm long and 3 cm wide. The bands were placed about 2 in. from the end of the strips to be immersed in the developing solvent. This solvent (di*iso*butyl ketone–acetic acid–water, 40:25:5) covered the bottom of the developing chamber to a height of about one inch. The developing chamber consisted of a 2 l. graduated cylinder or similar vessel fitted with a solid rubber stopper. Strips to be chromatographed were suspended in the chamber above the solvent for 30 min prior to development. The solvent was then allowed to ascend the strips by capillary action until it just met the upper edge of the lipid band. At this point, the strips were (1) removed from the chamber, (2) dried in an oven at 110°, (3) washed with water to remove the excess solvent, and (4) dried again at 110°. Following the second drying, the strips were (1) immersed in a saturated solution of Oil Red-0 in 60% aqueous ethanol for 1 hr, (2) rinsed with tap water, (3) oven-dried at 110°, and (4) scanned in the Spinco RB Analytrol.

Sensitivity and recovery studies were carried out in connection with the Oil Red-0 chromatographic procedure. An attempt was also made to evaluate this procedure by comparing the results obtained through its use with those derived by applying a modification of the method devised by TOURTELLOTTE *et al.* (1958*b*). In modifying TOURTELLOTTE's method for this purpose, plasma samples of known lipid content were used to replace the cholesterol standards, and the size of the CSF sample taken for assay was increased from 3 ml to 10 ml. All of the original manipulative steps were retained in the modified procedure, including precipitation and washing of the protein–lipid complex with trichloroacetic acid, followed by lipid extraction with alkaline ethanol (TOURTELLOTTE *et al.*, 1958*b*).

RESULTS AND DISCUSSION

Available methods for the separation of CSF lipids are generally acknowledged to lack the desired simplicity for routine use. Apparently, however, the intrinsic inability of these methods to remove quantitatively and selectively the lipids from CSF is not widely recognized (TOURTELLOTTE *et al.*, 1958*a*; HIRACH and AHRENS, 1958). The outcome of the present study should serve to focus attention on this fact which deserves careful consideration in biochemical studies of CSF lipids. As a preliminary step, the lipids must usually be removed to a more suitable environment for subsequent analysis or physicochemical evaluation. The ultimate success of such studies, therefore, depends in no small measure upon the efficiency and selectivity of the initial lipid extraction.

Any adequate procedure for extracting lipids from CSF must be accurate and also capable of precise execution under usual laboratory conditions. Preliminary studies were undertaken to determine which of the available procedures most nearly meets these requirements. The procedures selected for this purpose were dry extraction with hot and cold chloroform-methanol solvent and wet extraction with ethanol-ethyl ether and methanol–methylal mixtures, applied to 8–10 ml portions of CSF. Although the extractions were carried out with meticulous care, in no instance was the recovery of added serum lipids quantitative. Furthermore, none of the procedures evaluated seemed capable of obtaining CSF lipids completely free of contamination.

The most objectionable contaminants were the impurities held in coarse suspension that could not be completely removed by ordinary filtration or centrifugation. A positive biuret test was given by samples of this material isolated by ultracentrifugation

of lipid extracts at 246,500 g in a Spinco Preparative J Rotor. This suggests that this material may possibly consist chiefly of proteins precipitated in denatured form by the extraction solvents. These and other interfering contaminants were responsible for the introduction of an additional manipulative step. This entailed taking the initial lipid extracts to dryness and re-extracting the residue with a more specific lipid solvent like petroleum ether or benzene–amyl alcohol mixture.

Notwithstanding the objectional features of the extraction procedures studied, two of them gave reasonably satisfactory results: wet extraction with methanol–methylal solvent and dry extraction of freeze-dried fluid residues with boiling chloroform–methanol solvent in a multiple contact operation. These methods gave

TABLE 1.—COMPARISON OF OIL RED-O AND MODIFIED TOURTELLOTTE PROCEDURES

Procedure	No. of observations	Mean (mg/100 ml)	Standard deviation*	Relative error†
Oil Red-O	5	1·390	0·059	0·042
TOURTELLOTTE	5	0·982	0·134	0·136

* Standard deviation $= \sqrt{\frac{\sum_{n-1}^{n} (X_1 - \overline{X})^2}{n-1}}$ (where n is the number of observations; $\overline{X}$, the mean; and X_1, the individual determinations).

† Relative error $= \frac{\text{Standard deviation}}{\text{Mean}}$

essentially the same magnitude of yield of total lipid, and were comparable in precision. The use of boiling chloroform–methanol solvent, however, seems to be objectionable whenever it is desirable to preserve the chemical integrity of the CSF lipids. This can be expected to be particularly true of certain phospholipids that are likely to be susceptible to thermal alteration.

Strong evidence of this susceptibility was readily apparent. The extracts always acquired a yellow coloration when taken to dryness even under the mild conditions of 37° *in vacuo*. To show that this coloration was due to thermal effects, a number of extracts were taken to dryness in a make-shift freeze-drying apparatus. Essentially colourless residues were obtained in all of these experiments. Additional data to substantiate the thermal instability of certain CSF lipids are being uncovered in studies involving the chromatographic behaviour of CSF lipid extracts.

Attention will now be focused on a promising new analytical tool for the ultramicrodetermination of trace amounts of lipids: utilization of the complexing reaction of lipids with Oil Red-O to determine the total lipid content of CSF samples. A sample size of 8–12 ml was optimal in accomplishing this end. At this level, the new procedure gave a much higher yield of total lipids than a modified TOURTELLOTTE method. The former also seems at least as precise as the latter. These features of the new procedure are clearly reflected in the data of Table 1.

The precision attained with the modified TOURTELLOTTE method is somewhat less than might have been expected on the basis of previous reports (TOURTELLOTTE *et al.*, 1958*b*). The original TOURTELLOTTE procedure, in the hands of its inventors, was employed to acquire four separate sets of data. One of these had a relative error (TOURTELLOTTE's coefficient of variation) of 4·2 per cent. The relative errors noted

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for the other three were 5·9, 8·2 and 10·6 per cent. Two principal factors may have contributed substantially to the elevation of the relative error in the case of the modified procedure. One of these could have been a lack of sufficient practice with the method. The other could well have been the introduction of new, uncontrolled variants when the sample size was increased from 3 to 10 ml. These factors, however, would undoubtedly have operated to contribute coarse errors of the same order of magnitude to both procedures.

TABLE 2.—EVALUATION OF OIL RED-O PROCEDURE AT LOW CONCENTRATIONS

Peak no.	Volume CSF (ml)	Peak area (cm^2)	Lipid content (μg)
1	4·0	2·3 ± 0·025‡	20·1 ± 0·21
2	4·0	2·4 ± 0·023	22·8 ± 0·18
3	3·0	1·6 ± 0·016	14·1 ± 0·15
4	2·0	1·4 ± 0·016	11·0 ± 0·15
5	1·0	1·0 ± 0·008	9·0 ± 0·07
6	0·5	0·7 ± 0·006	5·8 ± 0·05
7	*	0·6 ± 0·008	5·0 ± 0·05
8	†	1·8 ± 0·020	15·0 ± 0·17

* 5 μg Plasma lipids (conversion factor)
† 5 μg Plasma lipids plus 2 ml of CSF
‡ Standard deviations based on four observations

The Oil Red-O procedure definitely seems to be very much more suitable for routine use than that of TOURTELLOTTE. For some purposes, it may even prove useful in dealing with much smaller initial volumes of fluid. The data in Table 2, included to illustrate this point, are based on the graphic integration of the areas under the curves in Fig. 1. Although the results presented in Table 2 are lacking in precision, they are in the right order of magnitude. The procedures underlying these results could undoubtedly be readily improved to give better precision, but there is a far more

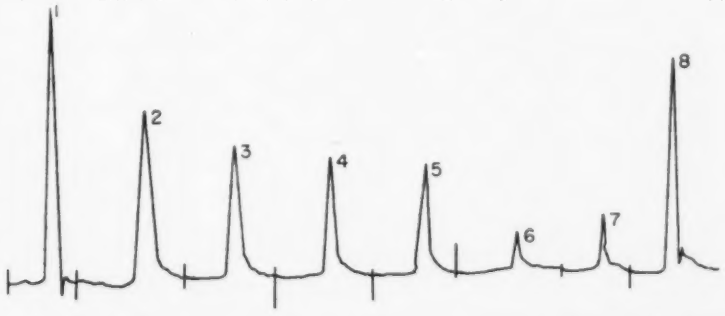

FIG. 1.—Spinco RB Analytrol scanning of lipid chromatograms stained with Oil Red-O. (The number in the parenthesis to the right of the one corresponding to the first six peaks represents the ml of cerebrospinal fluid taken for extraction.) Peak No. 7 represents 5 μg of plasma lipid, and peak No. 8, 2 ml of cerebrospinal fluid plus 5 μg of added plasma lipid.

urgent need for the development of more efficient methods of extraction from CSF. No analytical procedure for the quantitative estimation of these substances can be any better than the preliminary extraction upon which it is dependent. The same also holds true of the chromatographic detection of CSF lipids.

Preliminary chromatographic experimentation on silicic acid-treated glass paper strips has yielded highly promising results (MARINETTI, ERBLAND and KOCHEN, 1957; DIECKERT and REISER, 1956). This experimentation has clearly demonstrated that through the use of the method it is now possible to detect and classify individual CSF lipids as to chemical type provided they occur in concentration levels of at least 50 μg per 100 ml. In practice this can be accomplished without recourse to preliminary fractionation by extracting the lipids from 8 to 10 ml portions of CSF. This usually provides sufficient material for the preparation of several chromatograms. If some of these are developed unidimensionally in di*iso*butyl ketone–acetic acid–water (MARINETTI *et al.*, 1957) and others in *iso*octane solvent systems (DIECKERT and REISER, 1956), the former can be used for the detection of phosphatides and cerebrosides and the latter for nonphosphatides such as free cholesterol, esterified cholesterol, free fatty acids, and glycerides. As matters now stand, however, many improvements in the overall procedure are highly desirable.

Two particular advancements along this line would greatly extend the utilitarian value of the method. These are the development of more efficient methods for (1) selecting appropriate sample sizes to insure good chromatograms, and (2) resolving the lipids to be identified. On the whole, the chromatographic resolution attained with available solvent systems is poor. This is especially true of the phosphatide fraction.

An illustration of the poor chromatographic resolution encountered can be discerned from the tracings of typical chromatograms of methanol-methylal extracts of CSF (Fig. 2). The CSF used in preparing these chromatograms was obtained from patients believed to have no complaints involving the disintegration of neural tissue. The overlapping of spots B1 and B2 can be overcome by chromatographing the nonphosphatide fraction in the second dimension. Recourse to the two-dimensional approach, however, does not seem to help in dealing with the phosphatide–cerebroside fraction. Some suitable means for resolving this fraction prior to chromatographic analysis appears to offer the best solution to this problem.

Notwithstanding the difficulties discussed above, CSF lipids can now be chromatographically detected at levels as low as 5 μg per spot with Rhodamine G staining (DELSAL, 1944). Differential spot tests are generally applicable in minimal concentrations of the order of 5 μg per spot. At this level the phospholipid components of CSF were susceptible to wide variation. To this end, phospholipids were always chromatographically detectable but seemed to differ in number and kind from one fluid sample to another. Esterified cholesterol and neutral glycerides also always seemed to be present. Although it could not be demonstrated, these substances too may differ in kind from one CSF to another. Free cholesterol and cerebrosides could not always be revealed on the chromatograms. The same also appeared to hold true of the plasmalogens. Although an appreciable plasmalogen concentration has been reported (STAMMLER, 1954), no direct chromatographic evidence of the presence of these substances was found. The solvent fronts on some chromatograms developed in di*iso*butyl ketone–acetic acid–water, however, gave a positive stain with Schiff's reagent which was taken to be indicative of the presence of plasmalogens. Some of

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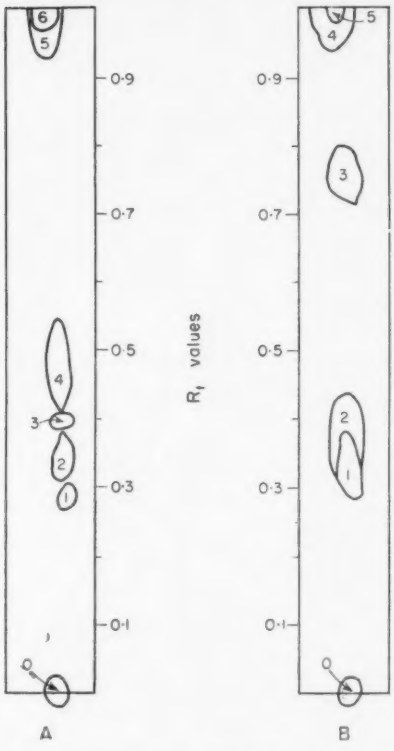

FIG. 2.—Tracings of original chromatograms of CSF phosphatides (A) and nonphosphatides (B). Chromatography was carried out on silicic acid-impregnated glass paper with di*iso*butyl ketone–acetic acid–water as the solvent system for phosphatides and *iso*octane–ethyl ether for nonphosphatides. Identification of the lipid spots, by methods described elsewhere (see text for references) is as follows: A—O = cerebrosides and probable traces of all other lipids; A—1, A—2, A—4, and A—6 = unidentified; A—3 = sphingomyelins; A—5 = cholesterol and its esters: B—1 = cholesterol: B—1,2 = triglycerides; B—3 and B—5 = unidentified; B—4 = cholesterol esters.

the unidentified phospholipid spots (Fig. 2) may possibly be cephalins or lecithins that have been reported by TOURTELLOTTE *et al.* (1958*b*) to occur in normal CSF at concentrations of 91 and 168 μg per 100 ml respectively. Suitable reference standards were not available for the chemical characterization of these unidentified spots as amine, choline, or inositol containing phospholipids. It was felt, therefore, that any attempt to classify them as to type solely on a basis of the more or less non-specific staining reactions would be too speculative.

A striking manifestation of the variability in the lipid 'profile' obtained by the chromatographic analysis of extracts derived from CSF becomes apparent when the tracing in Figs. 3 and 4 are compared with those in Fig. 2. Fig. 3 represents the chromatographically detectible phosphatides in a fluid from a patient suffering from multiple sclerosis. On the other hand, Fig. 4 was derived from a sample from a patient with diffuse sclerosis. In preparing the chromatograms from which these tracings were made, only one-fourth of the normal amount of material was used. Nevertheless, the quantity of cerebrosides and sphingomyelins revealed in the process was markedly

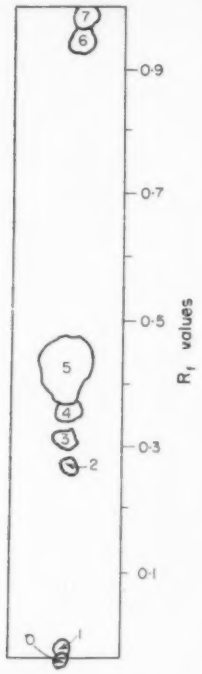

FIG. 3.—Tracing of a chromatogram representing phosphatide fraction of lipids extracted from CSF of patient diagnosed to have multiple sclerosis. Chromatography was carried out as previously described. Spots numbered 1, 2, 3, 5, and 6 are unidentified. Spot No. 0 = cerebrosides; No. 4 = sphingomyelins; and No. 7 = cholesterol and its esters.

increased. In contrast to these findings, no particularly unusual features of the nonphosphatide fractions were detected on chromatograms developed in the ethyl ether–*iso*octane system, with the exception of appreciable elevations of free cholesterol.

In view of the preceding considerations, it is apparent that chromatography on silicic acid-treated paper should prove extremely useful in detecting abnormalities in the lipid pattern of CSF. Although no attempt has yet been made to utilize the method to obtain quantitative data, this is not outside the realm of possibility. Since glass paper strips are employed in applying this method, the lipid spots on the chromatograms can be revealed by charring with sulphuric acid (MULDREY, MILLER and HAMILTON, 1959). A judicious combination of this process with densiometric scanning seems to offer promising possibilities of leading to eventual semi-quantitation.

Finally, an important factor to be taken into account in any analysis of CSF is the inadvisability of freezing and subsequently thawing of samples. Although this procedure was employed in the present study, it is fully realized that the sediment that forms in the process could possibly impair the validity of any lipid analysis. The material that settles out may remove lipids by adsorption, co-precipitation, occlusion, or it may actually be some form of lipid complex itself. An analysis of this material is contemplated when adequate analytical tools have been developed for this purpose. In the meantime, surface chemical studies of CSF to be reported at a later

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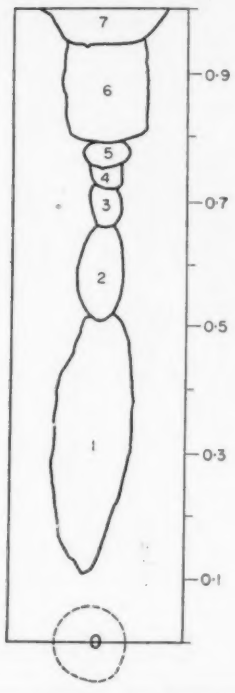

FIG. 4.—Tracing of a chromatogram representing phosphatide fraction of lipids extracted from CSF of patient diagnosed to have diffuse sclerosis. Spots numbered 1, 3, 4, 5, and 7 are unidentified. Spot No. 0 = cerebrosides; No. 2 = sphingomyelins; and No. 6 = cholesterol and its esters.

date reveal that the removal of the sediment that forms on thawing leads to a concomitant loss of surface-active CSF components.

SUMMARY

A simple and convenient chromatographic method has been developed for the quantitative determination of the total lipid content of cerebrospinal fluid. This method, in its present form, is applicable to 5—10 ml of CSF with an accuracy of 5 per cent or better. Another chromatographic procedure has also been evolved for the qualitative detection of the basic lipid types contained in the fluid. Through its use, the presence of neutral fat, cerebrosides, sphingomyelin, free cholesterol, and esterified cholesterol has been revealed in a preliminary fashion. Cephalins, lecithins, and plasmalogens, although undoubtedly present in the fluid, have not been positively identified.

Both of the chromatographic procedures developed during the course of this study are immediately useful on a more or less routine basis. Nevertheless, they still stand in need of further refinement. Research of this sort is now in process as a part of a long-range effort to find physicochemical measurements related to the detection of abnormalities in the content of surface-active materials occurring in trace amounts in CSF.

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HISTOCHEMISTRY OF MYELIN—I

PROTEINS AND LIPID–PROTEIN COMPLEXES IN THE NORMAL SHEATH

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THE histological and histochemical characteristics of the myelin proteins have attracted only little attention since the earlier observations of EWALD and KÜHNE (1874–1877). The neurokeratin resistant to proteolytic enzymes, identified chemically by KÜHNE and CHITTENDEN (1890), was thought to be located in the sheath and to be represented there by the protein network in myelin. BLOCK (1937) recorded that a histone-like protein could be extracted from the trypsin-resistant proteins of brain by treatment with acid. Little more was known about neurokeratin until it was shown by FOLCH and his colleagues (FOLCH and LEES, 1951; LEBARON and FOLCH, 1956) that two types of proteolytic enzyme-resistant proteins are present in the brain. One, a proteolipid protein, behaves as a lipid as regards its solubilities while the other, a trypsin-resistant protein residue (TRPR)† remains in the tissues after lipid extraction, but with lipid (diphosphoinositide) still attached to it.

The application of the solvent and enzyme-digestive methods used by FOLCH would appear to be the most suitable way to study these proteins histochemically in the nervous system. ADAMS (1957) recorded that the protein of peripheral myelin was strongly stained by the carboline method for tryptophan, while central myelin was more weakly stained. Later he observed that the protein of peripheral myelin and of the white matter of the CNS were resistant to digestion by trypsin and, therefore, were of the nature of neurokeratin (ADAMS, 1958). KOENIG (1959) identified proteolipid by extraction and by staining methods in the myelin of the CNS and the PNS. However, since it has been shown (FINEAN, HAWTHORNE and PATTERSON, 1957; FOLCH, LEES and CARR, 1958; ADAMS and TUQAN, 1961) that there is very little proteolipid in the sciatic nerve, KOENIG's identification of this protein in the peripheral sheath requires further investigation. WOLMAN (1958, 1960) has inferred from histochemical evidence that there is a trypsin-digestible protein in the myelin sheath of peripheral nerve. Digestion of this protein caused a diminution in the staining of the myelin sheath for lipids and polysaccharides.

The purpose of this investigation was to determine histochemically which of the trypsin-resistant proteins are constituents of the myelin of the CNS and of the PNS, and whether or not the proteins digestible by proteolytic enzymes (see WOLMAN, 1958) are different in central and peripheral myelin.

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† Abbreviations used in this paper are:
TRPR—Trypsin-resistant protein residue.
OTAN—Osmium tetroxide-α-naphthylamine
PNS—Peripheral nervous system.

METHODS AND RESULTS

Samples of human frontal cortex with its adjacent white matter, and sciatic nerve were obtained at autopsy. Only subjects believed to be free of neurological disease were chosen. Blocks were also prepared from the brains and sciatic nerve of the rat and mouse. The unfixed tissues were rapidly frozen in a mixture of solid CO_2 and acetone after which 6 μ sections were cut on the cryostat at $-20°$. A series of frozen sections from each tissue were subjected to one of the following treatments:

(a) extraction by chloroform–methanol (2:1 v/v) at 20° for 18 hr.
(b) digestion by 0·1% crystalline trypsin (Light's) in 0·15 M-phosphate buffer, pH 6·0, at 37° for 1–3 hr.
(c) extraction by phosphate buffer, pH 6·0, at 37°.
(d) digestion by 1% crystalline pepsin (Light's) in 0·15 M-phosphate buffer, pH 5·3, at 37° for 1–3 hr.
(e) extraction by phosphate buffer, pH 5·3, at 37°.

TABLE 1.—THE EFFECT OF DIGESTION AND EXTRACTION ON THE STAINING OF MYELIN LIPIDS AND PROTEINS IN HUMAN BRAIN AND SCIATIC NERVE

Treatment	*p*-DMAB and glycerol–$FeCl_3$ (tryptophan)		Alkaline tetrazonium (tyrosine, histidine and tryptophan)		Sudan black (lipid)	
	CNS	PNS	CNS	PNS	CNS	PNS
Control	+Fig. 1	++Fig. 4	++	++	++	++Fig. 7
$CHCl_3$—CH_3OH	−Fig. 2	++Fig. 5				
Trypsin pH 6·0	+Fig. 3	++Fig. 6	+		+	±Fig. 8
Buffer pH 6·0	+	++	++	++	++	++
Pepsin pH 5·3	+	++			+	+
Buffer pH 5·3	+	++			++	++

+ + +, + +, + = intensity of reaction ± = equivocal reaction − = negative reaction.

Sections from each extraction or digestion, together with an untreated control, were stained by the following methods: the *p*-dimethylaminobenzaldehyde–nitrite (*p*-DMAB) method for tryptophan (ADAMS, 1957), the glycerol–ferric chloride method for tryptophan (ADAMS, 1960*a*), the alkaline tetrazonium reaction for tyrosine, histidine and tryptophan (DANIELLI, 1947, 1950; PEARSE, 1953) and Sudan black for lipids (see Table 1 for results). Additional methods used were the thioglycollate–ferric ferricyanide method for cystine and cysteine (ADAMS, 1956), the performic acid–alcian blue method for cystine and cysteine (ADAMS and SLOPER, 1956), the Sakaguchi reaction for arginine (BAKER, 1947*a*) and the Millon test for tyrosine (POLLISTER, 1952).

Trypsin-digested sections of white matter and peripheral nerve showed a loss of lipid (see Table 1) which suggested that some of the myelin lipids were bound to an enzyme-digestible protein. Further histochemical tests were applied in order to show that this effect of trypsin was due to its proteolytic action and not to (a) its esterase

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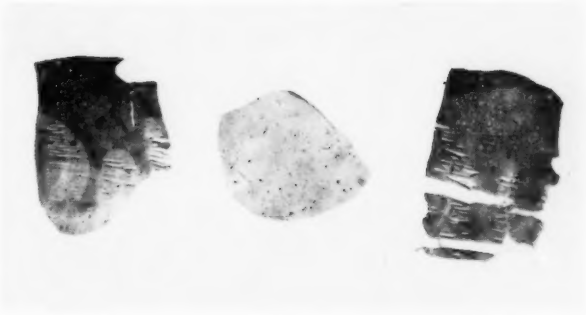

FIG. 1 FIG. 2 FIG. 3

FIG. 1.—Untreated human brain (frontal cortex and adjacent white matter). *p*-DMAB method for tryptophan, X3. Note staining of both grey and white matter. (The irregular cutting of the grey matter is an unavoidable defect of cryostat sections.)

FIG. 2.—As for Fig. 1 but extracted in chloroform–methanol. Much reduced staining of both grey and white matter.

FIG. 3.—As for Fig. 1 but digested by trypsin. Note reduction of staining in grey matter but unaltered reaction in white matter.

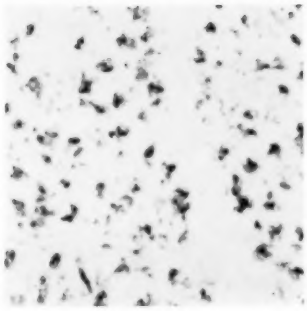

FIG. 4

FIG. 4.--Untreated human sciatic nerve. *p*-DMAB method for tryptophan, ×216. Note staining of 'neurokeratin' in the myelin.

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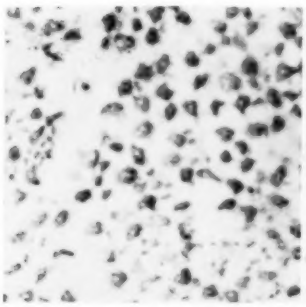


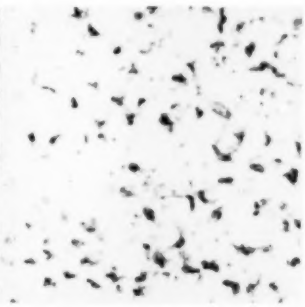
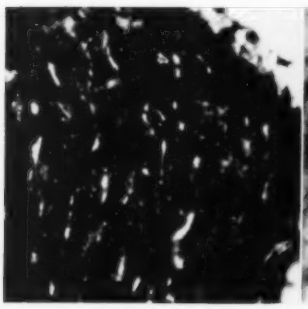


FIG. 5 FIG. 6

FIG. 5. (left)—As for Fig. 4 but extracted in chloroform–methanol. No alteration in staining of 'neurokeratin'.

FIG. 6. (right)—As for Fig. 4 but digested by trypsin. No alteration in staining.



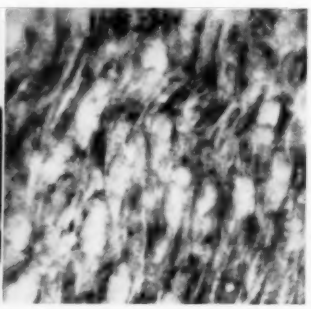


FIG. 7 FIG. 8

FIG. 7. (left)—Untreated human sciatic nerve. Sudan black, ×216.

FIG. 8. (right)—As for Fig. 11 but digested by trypsin. Note nearly complete loss of lipid from the myelin sheath.

action, (b) any containation of the trypsin preparation by lipolytic enzymes, or (c) a non-specific exchange of lipid between myelin proteins and another protein. Unfixed sections of CNS and PNS tissues, digested by trypsin or pepsin, were stained by the acid haematin method for phospholipids (BAKER, 1946, 1947*b*), by the ferric hydroxamate method for phosphoglycerides (ADAMS and DAVISON, 1959), by the osmium tetroxide-α-naphthylamine (OTAN) method for hydrophilic and hydrophobic lipid (ADAMS, 1959) and by the PAS reaction (modified after HOTCHKISS and MCMANUS; PEARSE, 1960). In both central and peripheral myelin there was a similar reduction in the staining of enzyme-digested tissues by these methods to that with Sudan black.

In order to determine whether or not the impurities of commercial trypsin would increase the extent or the rate of loss of lipid from myelin, further sections were incubated with a 1% solution of a commercial preparation of trypsin in phosphate buffer at pH 6·0. No such effect was found. Sections were also incubated with the 1% crystalline trypsin solution in the presence of a B-esterase inhibitor, Mipafox (10^{-3} M) (ALDRIDGE, 1954). The loss of lipid from these sections was as great as from those digested in trypsin alone.

In order to detect fatty acids liberated by either lipolytic or esteratic enzymes, sections were incubated in a 1% solution of crystalline trypsin in 0·4% $CaCl_2$. They were washed, treated with a 1% solution of $Pb(NO_3)_2$ and then immersed in a dilute solution of $(NH_4)_2S$ (modification of lipase method; GOMORI, 1952). Any liberated fatty acids should have been revealed by the deposition of black PbS but none in fact were set free by the action of trypsin.

Finally, unfixed sections were incubated for 1 hour in 0·1 and 1% solutions of casein in phosphate buffer at pH 6·0. This treatment did not remove lipid, stainable by Sudan black, from cerebral white matter.

In these experiments myelin was found to contain both trypsin-digestible and trypsin-resistant proteins. The resistant protein of CNS myelin has the characteristics of FOLCH and LEE's (1951) proteolipid, while that in peripheral myelin is akin to the TRPR isolated from whole brain by LEBARON and FOLCH (1956).

The liberation of lipid from sections treated with trypsin appeared to be due to the digestion of a protein moiety of a protein–lipid complex. This effect did not appear to be due to contamination of the trypsin preparation by lipolytic enzymes or to the esteratic rather than the proteolytic action of trypsin. Moreover, pepsin, which has a proteolytic but no esteratic action, had a similar though less marked effect to that of trypsin. The result of tryptic digestion did not appear to be a non-specific effect of protein as incubation in a buffered solution of casein had no effect on the subsequent staining of the myelin lipids.

DISCUSSION

Trypsin-resistant proteins (proteolipid and TRPR)

From the observations reported, it is clear that the trypsin-resistant protein of central myelin differs from that in peripheral myelin. Although there was no apparent difference in the histochemical amino acid patterns or enzyme digestibilities of these two proteins, yet the trypsin-resistant protein of the myelin of the CNS, by reason of its solubility in chloroform–methanol, is of proteolipid nature while the trypsin-resistant protein of peripheral myelin resists this solvent extraction and is, therefore,

akin to LEBARON and FOLCH's (1956) TRPR. KOENIG (1959), however, claimed that the protein (neurokeratin) of peripheral myelin was extracted by chloroform–methanol and was, accordingly, proteolipid. He did not confirm that the extracted substance was resistant to digestion by trypsin and pepsin. Moreover, KOENIG stained his sections by two methods (copper phthalocyanin and a mordant–haematoxylin technique) which probably react with lipid rather than protein (PEARSE, 1955, 1960; BAKER, 1946, 1947*b*; CAIN, 1947). Thus KOENIG's observations refer mainly to the extraction of lipid by chloroform–methanol and they have little or no direct relevance to the staining of myelin proteins.

TABLE 2.—AMOUNTS OF PROTEOLIPID AND TRPR IN PERIPHERAL NERVE AND CNS
(mg protein per 100 mg wet weight)

Tissue		Species	Proteolipid	TRPR
Peripheral nerve		horse[1]	0·063 (5)*	—
		bovine[2]	0·045	—
		rat[3]	0·075 (5)	0·55 (5)
Cord		rat[3]	0·77 (5)	0·41 (5)
Optic nerve		horse[1]	1·54 (4)	—
Brain	whole	mouse[4]	0·49	0·67
	whole	various[5]	—	0·38
	white matter	various[5]	2·0–2·5	—

* Figures in parentheses refer to the number of samples.
1 FINEAN, HAWTHORNE and PATTERSON, 1957.
2 FOLCH, LEES and CARR, 1958.
3 ADAMS and TUQAN, 1961.
4 FOLCH, 1955.
5 FOLCH and LEBARON, 1957.

The biochemical estimations of these proteins in the CNS and the peripheral nerve do not support KOENIG's conclusions. There is about ten times as much proteolipid in cerebral white matter, cord and optic nerve than in the peripheral nerve, while there is about the same amount of TRPR in both the CNS and PNS (see Table 2). The apparent absence of TRPR in the CNS, as judged by the only weak residual protein-staining of white matter after extraction in chloroform–methanol and digestion by trypsin, must be attributed to the dispersal and hence dilution of this protein throughout both grey and white matter (LEBARON and FOLCH, 1956). In contrast, TRPR can be recognised in peripheral myelin since it is concentrated in the sheath only.

Trypsin-digestible proteins

Nearly all the lipid matter in sections of CNS and peripheral nerve was extracted by chloroform–methanol. Suprisingly, however, treatment of unfixed sections with trypsin and pepsin also removed a large part of the lipid from myelin. This effect was

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probably due to the proteinase activity of these enzymes and not to the A-esterase action of trypsin (ALDRIDGE, 1956). The corresponding decrease in the intensity of staining by the tetrazonium method for tryptophan, tyrosine and histidine, in sections of white matter subjected to tryptic digestion, was not matched by a similar diminution in the staining of these digested tissues for tryptophan alone. This indicates that there is a trypsin-digestible protein in myelin which contains more histidine and tyrosine than the trypsin-resistant myelin proteins (proteolipid and peripheral nerve TRPR) but, conversely, less tryptophan. WOLMAN (1958) and WOLMAN and HESTRIN-LERNER (1960) showed that there is an enzyme-digestible protein in myelin. Removal of this enzyme-digestible protein caused a partial loss of lipids from the myelin, which suggests that there is a union between the two in the normal structure of the sheath.

TABLE 3.—THE CHARACTERISTICS OF LIPID–PROTEIN COMPLEXES IN MYELIN

Protein	Solubility in chloroform–methanol	Digestibility by trypsin	Associated lipids	Site
Proteolipid	soluble	resistant	phospholipid cerebroside	CNS
TRPR	insoluble	resistant	phospholipid	peripheral nerve
Enzyme-digestible protein	? insoluble	digested	phospholipid cerebroside	CNS and peripheral nerve

Lipid-protein bonds

The myelin sheath may be considered as a lamellar structure containing alternating bands of protein and lipid (see SCHMITT, 1957; ENGSTRÖM and FINEAN, 1958). These proteins and lipids are presumably arranged in such a way that, not only is structural rigidity imparted to the sheath by protein–protein bonds and by lipid–lipid bonds in the circumferential axis of the lamella, but also by protein–lipid bonds directed in a radial direction across the protein and lipid layers of each lamella unit. These lipid–protein bonds are probably hydrogen bonds or, alternatively, salt-linkages between phosphatidyl groups of phospholipid and free terminal amino-groups of protein. Such bonds probably account for the union between lipid and protein in the proteolipids, TRPR–lipid complex and in the trypsin-digestible protein–lipid complex. No matter what their exact physicochemical nature is, these bonds may be of considerable importance in stabilising the myelin sheath in a radial direction.

The lipids bound by proteolipid protein are phosphatides and cerebrosides (FOLCH and LEES, 1951), while only phosphatide is associated with TRPR (LEBARON and FOLCH, 1956). Phosphatide and cerebroside form at least a part of the lipid complex with the enzyme-digestible protein, since the specific staining of these lipids was less after tryptic and peptic digestion. The lipid–protein bonds of myelin, discussed in this paper, are summarised in Table 3.

From this investigation it was concluded that the trypsin-resistant protein of central

myelin (proteolipid) is different from that in peripheral myelin (TRPR), yet in both CNS and PNS there is a trypsin-digestible myelin protein. Digestion of this enzyme-sensitive protein caused a simultaneous loss of lipid from myelin. The significance of this to the process of demyelination will be the subject for further investigation (ADAMS and TUQAN, 1961).

SUMMARY

1. The nature of the proteins and lipid–protein bonding in myelin have been studied by histochemical means.

2. Of the trypsin-resistant proteins, proteolipid is a myelin constituent in the CNS while TRPR (trypsin-resistant protein residue) is the enzyme-resistant protein of peripheral myelin.

3. A trypsin-digestible protein is present in the myelin of both CNS and PNS. Digestion of this protein causes a simultaneous loss of lipid from the myelin sheath.

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HISTOCHEMISTRY OF MYELIN—II

PROTEINS, LIPID–PROTEIN DISSOCIATION AND PROTEINASE ACTIVITY IN WALLERIAN DEGENERATION

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THE process of demyelination in Wallerian degeneration may be pertinent to the aetiology of the human demyelinating diseases, since the study of this process may indicate where the causes of these diseases should be sought. As far as the myelin lipids are concerned, it has been shown that there are no chemical changes during the first week after nerve section, but thereafter the lipids are progressively degraded to cholesterol esters (JOHNSON, MCNABB and ROSSITER, 1949, 1950; ROSSITER, 1955). Likewise the histochemical characteristics of the myelin lipids remain unaltered during the first week of demyelination (NOBAK and MONTAGNA, 1952; NOBAK and REILLY, 1956) but in the second week, cholesterol esters accumulate in the degenerating sheath as is shown by the positive Marchi reaction of the myelin break-down products at this stage (ADAMS, 1958, 1960).

The early events in the first week of demyelination were attributed by ROSSITER and his colleagues to 'physical' disintegration of the sheath. However, although they showed that the myelin lipids were chemically unaffected in this preliminary phase no account was taken of changes in the myelin proteins or the bonding between lipid and protein in the sheath. It could be postulated that a protein constituent of myelin is chemically degraded during this early phase of demyelination or, alternatively, that there is a disruption of lipid–protein bonds, such as those of CNS proteolipid (FOLCH and LEES, 1951) or the trypsin-resistant protein residue (TRPR)† lipid complex of the PNS (TUQAN and ADAMS, 1961). In support of the former hypothesis, it was shown by TUQAN and ADAMS that proteolytic enzymes cause *in vitro* 'demyelination' apparently as a result of the liberation of lipid bound to a trypsin-digestible protein in the myelin of both CNS and PNS.

The purpose of this investigation was to determine by biochemical and histochemical means (a) whether there is any degradation of proteolipid protein and TRPR protein in degenerating myelin of the CNS and PNS, (b) whether there is disruption of the TRPR–lipid complex during demyelination and (c) whether proteolytic enzymes are present or become active in the degenerating nerve.

METHODS AND RESULTS

Biochemical investigations

Cordotomy or unilateral section of the sciatic nerve was carried out in rats: mice were subjected to unilateral or bilateral nerve section. After suitable periods the degenerating tissue was carefully dissected free and weighed on an aperiodic electric balance (sensitive to 0·1 mg). The cord samples

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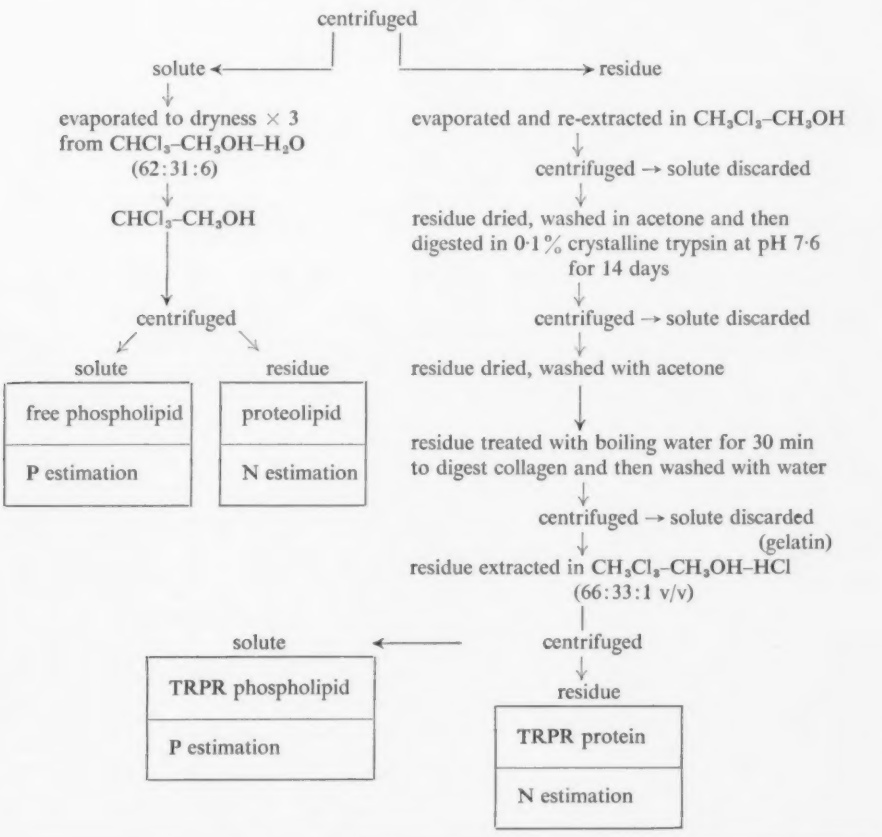
† Abbreviations used in this paper are: TRPR—trypsin-resistant protein residue.
PNS—peripheral nervous system.

weighed about 250 mg, and the distal segments of the divided nerves weighed 60–100 mg in the rat and 10–15 mg in the mouse. The following corrections for the increased wet weight of the degenerating nerve were made from the data of JOHNSON, MCNABB and ROSSITER (1949; 1950):

Days after section	Correction factor
0	×1·000
4	×0·746
6	×0·783
8	×0·820
12	×0·806
16	×0·793

Samples for the analysis of free phospholipid, proteolipid protein, TRPR protein and TRPR phospholipid were prepared according to the scheme in Table 1. Samples of mouse sciatic nerve for

TABLE 1.—PROCEDURE FOR PREPARATION OF PROTEOLIPID AND TRPR FROM NORMAL AND DEGENERATING SCIATIC NERVE AND CORD (MODIFIED FROM FOLCH AND LEES (1951) AND LEBARON AND FOLCH, (1956))



the estimation of tryptophan were digested in concentrated H_2SO_4 (Microanalar) for 30 min. Tissues for the estimation of proteinase activity were usually ground up in 0·02 N-HCl. In some experiments, however, tissues were prepared by soaking in acid without homogenization, by grinding, by hand homogenization, by repeated freezing and thawing, and by grinding followed by high speed centrifugation at 17,000 rev/min for 10 min.

The following analyses were made; lipid-bound phosphorus (see STRICKLAND, THOMPSON and WEBSTER, 1956), protein-bound nitrogen (micro-Kjeldahl), protein-bound tryptophan (method 5, PORTNER and HÖGL, 1953) and proteinase activity at pH 7·6 with casein as substrate (KUNITZ, 1946–1947; HERRIOTT, 1941).

TABLE 2.—CHANGES IN PROTEINS AND LIPIDS IN THE DEGENERATING SCIATIC NERVE

Days after section	Total phospho-lipid* (rat)	Proteolipid protein*† (rat)	TRPR protein*† (rat)	TRPR phospho-lipid* (rat)	Total phospho-lipid* (mouse)	Total protein tryptophan* (mouse)
0	5·20 ±0·48‡ (11)§	0·072 ±0·008 (5)	0·55 ±0·04 (5)	0·14 ±0·01 (5)	6·17 ±0·53 (8)	0·187 ±0·013 (8)
4					5·90 6·01 6·02	0·214 0·240 0·245
8	4·58 ±0·45 (5)	0·087 ±0·01 (5)	0·57 ±0·03 (5)	0·15 ±0·01 (5)	5·60 5·98 5·15	0·210 0·211 0·178
12					4·38 3·97 3·82	0·160 0·135 0·123
16	3·83 3·08	0·042 0·090	0·42 0·38	0·11 —	3·65	0·108

* mg/100 mg corrected wet weight.
† N × 6·25.
‡ Standard deviation.
§ No. of experiments or samples.

The analysis of proteins and lipids in normal and degenerating nerve and cord are summarised in Tables 2 and 3. Since most of the protein-bound tryptophan of peripheral myelin appeared to be in trypsin-resistant protein (TUQAN and ADAMS, 1961), estimations of this amino-acid were taken as an index of TRPR in the peripheral nerve. The activity of proteolytic enzymes in ground-up samples of normal and degenerating, nerve is recorded in Table 4. Comparative figures for proteolytic activity after soaking grinding and grinding plus centrifugation (sediment and supernatant together) are presented in Table 5. The activity of samples prepared by hand-homogenization and by repeated freezing and thawing were no greater than that of ground-up samples.

From these results it may be deduced that there was no chemical change in the trypsin-resistant proteins or TRPR–lipid complex during the first 8 days after nerve section. Later these proteins and lipid-complexes decreased in step with the degradation of the free phospholipids. Although considerable activity of proteolytic enzymes was

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TABLE 3.—CHANGES IN PROTEINS AND LIPIDS IN THE DEGENERATING RAT CORD

Days after section	Total phospholipid*	Proteolipid protein*†	TRPR protein*†	TRPR phospholipid*
0	5·25 ± 0·35‡ (11)§	0·77 ± 0·08 (5)	0·41 ± 0·04 (5)	0·24 ± 0·02 (5)
8	4·97 ± 0·10 (5)	0·69 ± 0·05 (5)	0·36 ± 0·04 (5)	0·39 ± 0·06 (5)
16	3·40 2·38	0·62 0·54	0·33 0·30	0·21 0·34

* mg/100 mg corrected wet weight.
† N × 6·25.
‡ Standard deviation.
§ No. of experiments.

TABLE 4.—PROTEINASE ACTIVITY IN THE DEGENERATING SCIATIC NERVE OF THE MOUSE
Samples were prepared by grinding and activity is expressed as percentage of that in the contralateral normal nerve

Days after section	0	1	2	3	4	6	8	12	16
Activity	100	127 128	117 149	124 178 222	93 163 151 217 160	117 150	108 108 155	106 112 159	120 134 200
Mean	100	128	133	175	157	134	124	126	151

TABLE 5.—COMPARISON OF THE EFFECT OF DIFFERENT EXTRACTION METHODS ON THE OBSERVED PROTEOLYTIC ACTIVITY OF SAMPLES OF NORMAL AND DEGENERATING MYELIN
(μg tyrosine/100 mg corrected wet weight/hr)

Extraction method	Days after section	Proteinase activity	
		normal nerve	degenerating nerve
Soaked nerves	4 4	222 258	148 265
Ground-up nerves	4 4 3	255 186 207	385 405 370
Ground-up and centrifuged nerves	4 4 3	335 343 292	364 384 334

noted in the normal nerve, the activity of this enzyme increased from the first day after section onwards in ground-up samples of degenerating nerve (see Table 4). Since much greater amounts of enzyme were recovered from the normal nerve after separation of the components by centrifugation (see Table 5), it was concluded that the increased enzymic activity of ground samples of degenerating nerve was mainly due to the *in vivo* 'liberation' of enzyme from the nerve already partly disrupted as a result of the demyelinating process. This view is supported by the observation that there was little difference in the proteinase activities of normal and degenerating nerve when both had been subjected to high speed centrifugation (see Table 5).

Histochemical investigations

Samples of degenerating cord and sciatic nerve were taken from the animals used for the biochemical investigations. Some tissues were cut unfixed on the cryostat at 6 μ while those for the study of proteinases were briefly fixed in 4% formaldehyde–saline for 2 hr and cut on the freezing microtome at 15 μ. Sections of every tissue were stained by the OTAN method for hydrophilic and hydrophobic lipids (ADAMS, 1959) and by the *p*-DMAB method for tryptophan (ADAMS, 1957). Some sections were also stained by haematoxylin and eosin, Sudan IV, 0·5% trypan blue in 0·15 M-phosphate buffer at pH 8·0. Proteolytic acitvity was demonstrated by mounting sections of briefly fixed tissues on blackened photographic plate and incubating the preparation in a closed humid atmosphere in saline at 37° for 1 hr (see ADAMS, FERNAND and SCHNIEDEN, 1958; ADAMS and TUQAN, 1960). The sites of gelatin digestion (i.e. proteolytic activity) were revealed as clear areas where the silver granules had been washed out. The sensitivity of the method is such that an area of digestion, 30 μ in diameter, would result from the action of the order of 1 $\mu\mu$g of trypsin.

TABLE 6.—HISTOCHEMICAL CHANGES IN DEGENERATING NERVE AND CORD

Days after section	Hydrophilic lipid (OTAN red)		Hydrophobic lipid (OTAN black)		Lipid–protein dissociation,		Basophilia (trypan blue)	Proteolytic activity
	nerve	cord	nerve	cord	nerve	cord	nerve	nerve
0	++Fig. 1	++	−Fig. 1	−	−Fig. 5	−	+Fig. 5	+ Fig. 7
4	++		−		±		+–++	++Fig. 8
6	++		−		+		+++	++
8	++Fig. 2	++	±Fig. 2	−	++Fig. 6	−	+++Fig. 6	++
12	++Fig. 3		+Fig. 3		++		+++	++
16	±Fig. 4	±	++Fig. 4	++	++	−		

+ + +, + +, + = intensity of reaction ± = equivocal reaction — = negative reaction

The results are summarised in Table 6. The dissociation of lipid from protein in the degenerating peripheral nerve indicated that there had been some disturbance in lipid–protein bonds so that lipid had come to lie free within the sheath. The increased basophilia of the proteins in degenerating myelin, as shown by trypan blue, indicates either that the properties of these proteins had been altered by digestion or denaturation or, alternatively, that protein amino-groups had become exposed after disruption of

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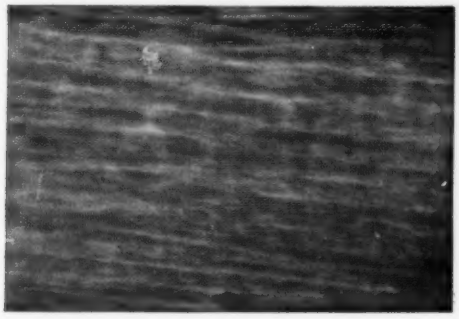

FIG. 1. (×336)

FIG. 2. (×336)

FIG. 3. (×336)

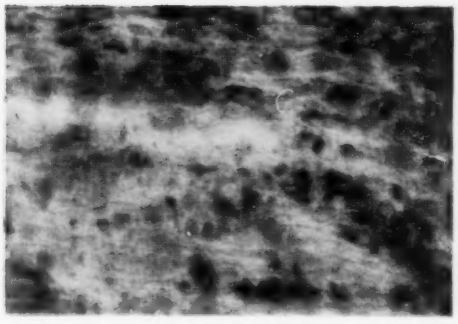

FIG. 4. (×336)

FIG. 5. (×840)

FIG. 6. (×840)

FIG. 1.—Normal mouse sciatic nerve. OTAN method. The hydrophilic lipids of normal myelin are stained red.

FIG. 2.—Mouse sciatic nerve 8 days after section. OTAN. Note the fragmentation of the sheath but the lipids retain their normal staining characteristics.

FIG. 3.—Mouse sciatic nerve 12 days after section. OTAN. Hydrophobic cholesterol esters in the degenerating sheath are stained black but hydrophilic lipids, stained red, are still present.

FIG. 4.—Mouse sciatic nerve 16 days after section. Hydrophilic lipids have by now disappeared. OTAN.

FIG. 5.—Normal mouse sciatic nerve. Trypan blue and Sudan IV.

FIG. 6.—Mouse sciatic nerve 8 days after section. Trypan blue and Sudan IV. Lipid-protein dissociation is prominent. Note that the myelin proteins have become more basic and are stained more strongly than in the normal nerve (Fig. 5).

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lipid phosphatidyl groups bound to them. Proteinase activity was also increased at a correspondingly early stage of demyelination. By inference, this suggests that enzyme-sensitive protein was being digested in the degenerating myelin.

DISCUSSION

Proteins and lipid-protein bonds in Wallerian degeneration

There was no significant decrease, detectable by chemical means, in the amount of TRPR by the 4th and 8th days after section of the peripheral nerve, or of proteolipid by the 8th day after cordotomy. Likewise there was no decrease in the amount of lipid, as phospholipid, bound by these proteins. By the 16th day after section the amount of these proteins and the corresponding protein-bound lipids had decreased in step with the fall in lipid not bound to protein. The fall in these proteins, protein-bound lipids and free lipid thus takes place during the second phase of demyelination—ROSSITER's (1955) stage of chemical degradation of the myelin lipids. In the histochemical study of the degenerating peripheral nerve dissociation of lipid from protein became apparent only 6 days after nerve section, yet there was no decrease in the amount of stainable protein and lipid in the disrupted myelin. By the 8th day this process of dissociation had become more prominent.

During this early stage of degeneration there was a very substantial increase of available basic groups in the myelin protein, as shown by staining with trypan blue. This was possibly a result of increased accessibility of protein-bound amino-groups previously masked by salt-linkage with the phosphatidyl group of phospholipid. In parentheses, it should be noted that WOLMAN (1957) detected an increase of acidic groups in the early stages of demyelination which he attributed to the liberation of acidic mucopolysaccharide from the degenerating sheath.

In central myelin, however, lipid–protein dissociation could not be detected at any period between the 8th and 16th days after cord section. The failure to detect this dissociation in central myelin was probably due to the weak staining of these myelin proteins with the result that protein could be only poorly defined.

The OTAN (modified Marchi) reaction, however, had become positive (OTAN-black) by the 12th day in both central and peripheral myelin, indicating the formation of cholesterol esters during the phase of chemical degradation of the sheath (ADAMS, 1958, 1960).

Proteolytic enzymes in Wallerian degeneration

It was apparent from the observations reported by TUQAN and ADAMS (1960) that digestion of the trypsin-sensitive protein in myelin leads to 'demyelination' *in vitro*. Since proteolytic enzymes were detected biochemically and histochemically in normal sciatic nerve and their apparent activity somewhat increased during Wallerian degeneration, it can reasonably be inferred that these enzymes play an important role in the demyelinating process. Moreover, the increased activity started in the early stages of demyelination, from the first day onwards. The digestion of the trypsin-sensitive moiety of the myelin proteins would, however, be difficult to detect chemically, since it may well be masked by the increase of cellular protein or be overshadowed by the loss of protein from the degenerating axon.

It seems likely that the increased proteolytic activity of the degenerating nerve is

mainly due, not to increased cellular secretion, but to liberation of enzyme pre-existing in or around the normal nerve. Complete disruption of normal myelin by high-speed centrifugation, in contrast to imperfect 'homogenization' by grinding, liberated as much proteinase as was found during demyelination. The increased proteolytic activity of the degenerating nerve, in samples 'homogenised' by grinding and in the histochemical preparations, may therefore reflect the 'liberation' or greater accessibility of these enzymes during demyelination. In this connection CHAPMAN and WOLFF (1959) found a substantial increase of CSF proteolytic activity in diseases where there had been active or recent necrosis of brain tissue.

A cathepsin, most active at pH 3·8, and a neutral proteinase have been identified in the brain (ANSELL and RICHTER, 1954*a*,*b*; RICHTER, 1959). MCCAMAN and ROBINS (1959) have reported a two to three-fold increase in peptidase activity in the degenerating tibial nerve at 14 days after section and a more delayed response in the sectioned optic nerve. These findings are not exactly comparable to those recorded here, since they were made 2 weeks after nerve section and not during the early stages of demyelination. VOGEL (1951) found that demyelination resulted from the intravenous or intracerebral injection of purified lipase while the introduction of proteolytic enzyme into the brain by the latter route lead to focal massive necrosis as well as demyelination. Although the relevant controls were made, it is doubtful whether intracerebral injection is a suitable way to test myelinolytic agents, since it could be postulated that oedema resulting from trauma had caused any demonstrable demyelination.

From the observations in this paper the following conclusions can be drawn:

(1) There is no significant change, detectable by chemical analysis, in the amount of proteolipid protein, TRPR or of the lipid bound by TRPR during the preliminary phase of physical disintegration in degenerating central and peripheral myelin. Subsequently, during the phase of chemical degradation, the levels of these proteins and protein-bound lipids fall *pari passu* with the fall in the free lipids, as was found by ROSSITER and his colleagues.

(2) Lipid–protein dissociation occurs in the peripheral nerve during the first week of demyelination. Since, by chemical analysis, it was shown that there was no loss of lipid bound to trypsin-resistant protein (TRPR), this dissociation must involve rupture of bonds between lipid and proteolytic enzyme-digestible protein.

(3) Proteolytic enzymes were detected in the normal peripheral nerve. Since their activity increased in the preliminary phases of Wallerian degeneration, the release of of these enzymes *in vivo* may be an important initiating factor in demyelination. However, this increased proteinase activity in the degenerating nerve is more probably due mainly to the liberation of enzyme from the myelin sheath or axon than to increased cellular synthesis. Moreover, the onset of increased enzyme activity occurs well before the cellular proliferation and infiltration described by ABERCROMBIE and JOHNSON (1946) and by CAVANAGH and WEBSTER (1955).

The digestion of the enzyme-sensitive protein from myelin would free the lipid attached to it. Such freed lipid would then appear in histological and histochemical preparations to have become 'dissociated' from the surviving undigested trypsin-resistant protein of myelin. This postulated mechanism would account for ROSSITER's (1955) preliminary stage of disintegration of the sheath before the myelin lipids are chemically degraded. It must be emphasized, however, that the action of a proteolytic

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FIG. 7.—Proteolytic activity in normal mouse sciatic nerve. Gelatin film method, ×96.

FIG. 8.—Increased proteolytic activity in mouse sciatic nerve 4 days after section. Gelatin film method, ×96.

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enzyme on the myelin sheath may only be one among a number of factors concerned in the early stages of the demyelinating process.

SUMMARY

1. Trypsin-resistant myelin proteins, lipid-protein bonds and proteinase activity have been studied in Wallerian degeneration by histochemical and biochemical means.

2. Proteolipid, trypsin-resistant protein residue (TRPR) and the TRPR–lipid complex do not undergo significant change during the early phase (first week) of demyelination.

3. During this preliminary phase of demyelination, liberation of proteolytic enzymes occurs, so that there is an increase of proteinase activity in the degenerating peripheral nerve. In this early phase, myelin lipid becomes dissociated from the protein of the sheath. This dissociation was not observed, however, in degenerating myelin of the CNS.

4. It is suggested that digestion of the enzyme-digestible protein of myelin frees bound lipid so that lipid and the surviving trypsin-resistant protein appear to be 'dissociated' from each other in the early stages of demyelination.

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THE SEPARATION OF THE CYTOPLASMIC PROTEINS OF BRAIN BY ELECTROPHORESIS IN A STARCH GEL MEDIUM

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THE applications of electrophoretic techniques to the analysis of proteins from cerebral tissues or cerebrospinal fluid have been increasingly developed within the past few years, and attempts have been made to correlate changes in the quantities and distributions of the various protein fractions with changes in the physiological state of the tissue (for summary of literature, see LOWENTHAL, KARCHER and VAN SANDE, 1959; KARCHER, VAN SANDE and LOWENTHAL, 1959).

Of the methods employed, the majority have made use of electrophoresis on paper of extracts of cerebral tissues prepared either by direct extraction with buffered salines (KAPS, 1954; HOFMANN and SCHINKO, 1956; ROBERTSON, 1957; see LE BARON, 1959) or by extraction after treatment with mixtures of ether and ethanol (KEUP, 1955). Such methods of extraction necessarily bring into solution proteins from all the structures of the cell and alternative methods employing isotonic sucrose to obtain only the cytoplasmic proteins have been preferred by other workers (CARAVAGLIOS and CHIAVERINI, 1956; LOWENTHAL *et al.*, 1959). With few exceptions the resulting extract has then been further treated either by freezing or by dialysis against distilled water, and insoluble material, formed during this treatment, removed before subjecting the preparations to electrophoresis. Even so, the protein separations achieved have been recognised as being far from satisfactory (ROBERTSON, 1957; LOWENTHAL *et al.*, 1959).

When the present work was begun the recently introduced technique of electrophoresis of serum proteins in a starch gel medium (SMITHIES, 1955; ASHTON, 1957) presented itself as a possible means of achieving separations of a greater degree of clarity than those obtained by the use of a paper medium. It was therefore decided to examine such a technique as applied to the separation of cerebral proteins. For ease of working, the proteins soluble in isotonic sucrose were chosen since they comprise the constituents of a definite cellular compartment, the cytoplasm, and also avoid complications due to the presence of additional proteins from different cellular structures.

Since this work began the use of an agar gel medium has been shown to be advantageous over paper (KARCHER *et al.*, 1959). Nevertheless, the separations achieved with a starch gel appear to be superior to those obtained with agar and are presented below.

MATERIALS AND METHODS

Materials

Potato starch (Batch No. 023348) and chemicals (analytical reagent grade) were supplied by Hopkin and Williams Ltd., Chadwell Heath, Essex. Naphthalene Black 10B was supplied by G. T. Gurr Ltd., London, S.W.6.

Methods

Preparation of cerebral extracts. Rats, mice and guinea-pigs were sacrificed by a sharp blow on the neck, and pigeons by decapitation. In all cases the skull was opened, the dura displaced, and the whole brain removed as rapidly as possible. Ox brains were obtained from the Metropolitan Cattle Market immediately after slaughter of the animals and were stored in polythene bags packed in crushed ice during transportation to the laboratory. Excess blood was removed from the surface of the brain by blotting with filter paper. The whole brain, or requisite portion, was then dispersed in a thick walled glass tube fitted with a plastic pestle (KAMPHAUSEN and MORTON, 1956) using 9·0 ml of aqueous sucrose (0·25 M) for each 3·5 g wet weight tissue. No intact cells were visible in samples of dispersions when they were viewed with a phase-contrast microscope. The dispersion was centrifuged for 30 min at 104,000 **g** using the No. 40 head of the Spinco Model L ultracentrifuge. The supernatants so obtained were clear when viewed in transmitted light. The residue was discarded. The supernatants were poured into dialysis tubing, inflated diameter 0·25 in. (Visking Ltd.), and subjected to a current of air from a fast-running fan situated about 18 in. from the samples for 6–7 hr at 2° in a cold room. This procedure resulted in an approximately ten-fold concentration of the original supernatant. The final concentration of protein was 60–80 mg/100 ml. The solution was clear after concentration and did not throw a precipitate. In experiments in which young guinea-pigs (80–100 g body wt) were sacrificed by freezing in liquid nitrogen the procedure was essentially similar to that described by KRATZING and NARAYANASWAMI (1953). After the frozen tissue had been finely ground under liquid nitrogen in a Wedgewood mortar it was dispersed in chilled aqueous sucrose (0·25 M) and centrifuged as described above.

Preparation of starch. Potato starch was partially hydrolysed by a procedure essentially similar to that described by SMITHIES (1955). Thus starch (300 g), acetone (600 ml), and concentrated hydrochloric acid (6 ml) were incubated together for 45 min at 37·5°, all reagents and glassware having been previously equilibrated to 37·5°. The hydrolysis was stopped by the rapid addition of chilled M-aqueous sodium acetate (150 ml). The suspension was immediately filtered with suction through two sheets of Whatman No. 1 filter paper and the starch cake washed thoroughly with distilled water (5 l.). The starch was then resuspended in distilled water (500–600 ml) and allowed to stand overnight at room temperature. It was then refiltered and washed with distilled water (1 l.) and acetone (500 ml). Finally it was dried at 37·5° and 7 or 8 such preparations were thoroughly mixed to provide a working preparation. This procedure was adopted to eliminate any slight variations between the individual batches.

Preparation of gels and electrophoresis. The required amount of partially hydrolysed starch was heated for 95 sec with buffered solution (100 ml) containing either 0·025 M or 0·04 M-borate at pH 8·3. The gels were cast in moulds made of perspex (I.C.I. Plastics Division) measuring internally 24 × 4 cm with a depth of 0·7 cm, which had been thinly smeared with liquid paraffin. Sufficient gel was poured, in one rapid movement from one end of the mould to the other, to yield a slight excess. Gels were immediately covered with sheets of polythene (I.C.I. Plastics Division) 0·0625 in. thick which were also smeared with paraffin. A heavy glass plate and weight were placed on top of the polythene in order to extrude the excess gel from the mould. The gels were allowed to cool to room temperature before being equilibrated to 2° in a cold room. They were used 16–18 hr after pouring. A strip of Whatman E.17 paper 0·7 cm wide was cut to the required length and soaked in the sample before being introduced into a vertical slot cut in the gel. The slot was 9 cm from the cathode end of the gel. The gel was then covered with a thin sheet of polythene to prevent evaporation during electrophoresis. Horizontal electrophoresis was carried out in an 'Electrophoresis sample preparation tank' (Evans Electroselenium Ltd.). Single strips of Whatman E.17 paper soaked in the buffer were used to connect the ends of the gels to the buffer compartments. A voltage gradient of 8 v/cm was applied for 6 hr; the temperature of the gels being kept as low as possible by performing the separation at 2° in a cold room. After electrophoresis the gels were placed in a cutting guide similar to that

described by SMITHIES (1955) by inverting the moulds over the guide and gently easing out the gel with a broad spatula. The sample paper was removed and the gel covered with a strip of polythene and a glass plate upon which was placed a 2 kg weight. Any air trapped between the gel and the cutting guide was carefully pressed out before the glass plate was put in position. The gel was then cut by drawing a taut, thin wire, in one continuous movement across the top of the guide. The glass plate and weight were removed, the cutting guide inverted, and the gel eased out onto the polythene. The two halves of the gel were separated and placed in a shallow glass tray, cut faces uppermost. The slices were stained for 5 min in Naphthalene Black 10B (1% w/v) dissolved in a mixture of methanol, water and glacial acetic acid (75:25:10 v/v). The background was eluted with a mixture of methanol, water and glacial acetic acid (50:50:10 v/v) until only a very pale blue coloration remained. The solution was renewed frequently during the elution. The results were recorded by freehand drawing from the original gels which were placed on a ground glass screen illuminated from below by a tungsten filament lamp. When required, gels were photographed in incident light from two 500 watt photoflood lamps. Kodak B.40 plates were used without any filter. Attempts to stain for lipo-proteins by means of Oil red O, and Sudan Black (G. T. Gurr Ltd.) were unsuccessful.

Estimation of proteolysis in cerebral extracts. The supernatant obtained by centrifuging the dispersion was divided into two portions. The nitrogen content of one was estimated immediately, the other being put through the concentration procedure and diluted back to its original volume before being estimated. Each portion was divided into two parts. One part was used for the estimation of total nitrogen. An equal volume of 20% (w/v) aqueous trichloroacetic acid (TCA) was added to the other part. After centrifuging, the supernatant was decanted and the precipitate washed twice with 10% (w/v) aqueous TCA. The washings were added to the original supernatant. The nitrogen contents of the precipitate and supernatant were then determined.

Determination of protein. This was determined by the method of LOWRY *et al.* (1951) using crystalline bovine serum albumin (L. Light and Co. Ltd.) as the standard.

Determination of nitrogen. This was determined by the method of CHIBNALL *et al.*, (1943). The final distillation was carried out in a MARKHAM (1942) still, with boric acid (CONWAY, 1947) as the trapping agent.

RESULTS

Factors involved in the electrophoretic separations

Initial experiments using the conditions described by SMITHIES (1955) to separate serum proteins, showed that these were inapplicable to extracts of cerebral proteins. Under such conditions little or no separation of the proteins was obtained. Investigation of the conditions necessary to obtain a separation of the various protein fractions showed that the most suitable were those described in detail above. The possible variables investigated were: type of buffer, concentration and pH, and the percentage of starch by weight in the final gel. Buffer systems tested and found to give poor resolution were, veronal–acetate, pH 8·6; veronal–citrate, pH 8·6; phosphate, pH 7·8; 2-amino-2-hydroxymethyl-propane-1:3-diol, pH 9·5; acetate, pH 5·0; and the discontinuous buffer system of POULIK (1957). Borate was found to be the most effective when used at two concentrations. In gels made with borate (0·025 M), the proteins migrating to the cathode were best separated, while those migrating to the anode were best separated in gels made in borate of 0·04 M concentration. Variation of the pH from 8·0 to 9·0 showed that a buffer of pH 8·3 was the most suitable for use with starch prepared as described. Under these conditions the final pH of the gel was 8·0. Study of the percentage of starch required in the gel led to the use of a concentration of 12% (w/v). The suitability of each of these factors was judged by the improvement in the degree of resolution of the extracts. Using the above conditions in borate buffer, some 10–15 protein bands could be detected according to the extract studied. To obtain greater reproducibility it was found advantageous to standardise the time of heating during the preparation of the gel. It has been found necessary to use

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batches of starch which have been prepared and stored for not longer than three months. Longer storage leads to a gradual decrease in the sharpness of the separations.

Assessment of proteolysis during sample preparation

It has been shown (ANSELL and RICHTER, 1954) that cerebral tissues contain proteolytic enzymes capable of rapidly degrading cerebral proteins when cell structure is destroyed. Since such action could lead to a marked alteration in the proteins extracted, attempts were made to estimate the extent, if any, of proteolysis during the period of preparation of the sample. In extracts prepared as described above, the total nitrogen precipitated with TCA was determined immediately after obtaining the extract and again after pervaporation, together with the nitrogen not precipitable by TCA. The results of these experiments (Table 1) showed that little, if any, proteolysis

TABLE 1.—ESTIMATION OF PROTEOLYSIS DURING PREPARATION OF CEREBRAL EXTRACTS FOR ELECTROPHORESIS BY CONCENTRATION IN DIALYSIS TUBING

Whole guinea pig brain was extracted with 0·25 M-aqueous sucrose. Dispersions were centrifuged at 104,000 g for 30 min. Nitrogen was estimated as described in Methods. Results are expressed as μg nitrogen ± standard deviation of the mean. The number of estimations is in parentheses.

Experiment No.	Before concentration			After concentration		
	Whole	TCA insoluble	TCA soluble	Whole	TCA insoluble	TCA soluble
1	228 ± 0·86 (3)	162 ± 1·81 (3)	68·6 ± 3·64 (3)	224 ± 2·36 (3)	161 ± 0·86 (3)	69·9 ± 3·87 (3)
2	287 ± 7·0 (3)	220 ± 5·15 (3)	51·1 ± 1·22 (3)	277 ± 5·5 (3)	223 ± 1·55 (3)	58·3 ± 6·65 (3)

took place during the concentration procedure. A comparison was also made between cerebral extracts prepared from animals sacrificed as described in Methods and from animals frozen in liquid nitrogen. After separation by electrophoresis no qualitative difference was detectable between the two preparations. It is realised that these results do not rule out rapid changes occurring during the initial few minutes in the preparation of the extract but show that such changes are not persistent and that the method of sacrificing the animal has little or no effect upon the final electrophoretic patterns obtained.

Comparison of proteins extracted from different species and parts of brain

Extracts of whole guinea-pig, rat or pigeon brain, when separated in borate buffered gels, yielded patterns similar to those shown in Fig. 1. This figure is a photograph of separations of extracts made from the whole brain of the pigeon and the guinea-pig. The different separations achieved with the two concentrations of buffer can be clearly seen, as can major differences in the mobilities of the proteins present in each extract. Differences involving minor components are not readily reproduced by photography but are better revealed by viewing in transmitted light. A reproduction of the gels shown in Fig. 1 is presented in Fig. 2 (patterns 1 and 2). Reproductions were made with measurement and drawing to the full scale of the patterns seen when gels were viewed as described above. The background staining is considered to be due to unresolved components and was generally less severe than would appear from the photograph. Although it could be reduced by staining for brief periods of 1 min, this resulted in a loss of definition amongst fractions present

in small quantities. Also in Fig. 2 are presented comparisons of the patterns obtained from rat and guinea-pig and from three strains of mice (see below).

The bands are not due to contamination with serum. Under the conditions employed for obtaining the brains, the cerebral content of blood varies from 2–6 per cent (VLADIMIROV, 1955). When guinea-pig serum was added at a concentration of 6% to extracts of cerebral proteins from guinea-pig brain little if any changes were noted in the various patterns, only the band corresponding to the position reached by serum albumin showed a slight increase in intensity. Serum alone at this concentration was largely unfractionated under the conditions used, only the albumin being clearly detectable.

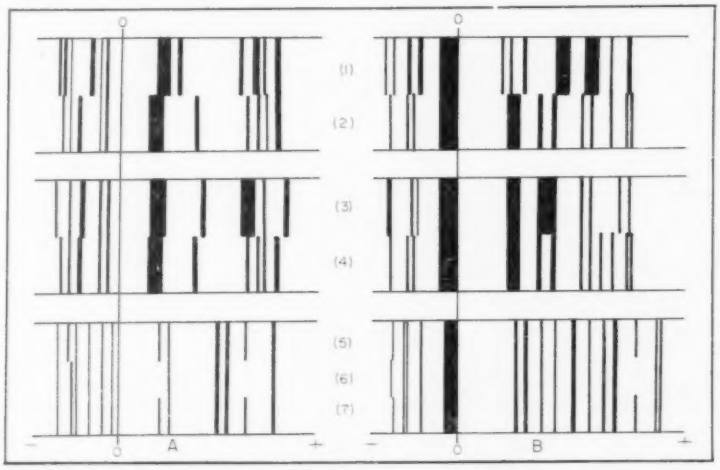

FIG. 2.—Diagrammatic representation of starch gels after electrophoresis of soluble proteins extractable from whole brains of various animals.
Gels in column A made with 0·025 M-borate buffer, pH 8·3. Gels in column B made with 0·04 M-borate buffer, pH 8·3. 'O' indicates the point of insertion of the samples. (1) pigeon, (2) guinea pig, (3) rat, (4) guinea pig, (5) albino mouse, (6) C57 black mouse, (7) CBA mouse.

When extracts prepared from different parts of the brain were examined marked differences were seen in the patterns obtained (Fig. 3). This was particularly noticeable in extracts of human cortex and cerebellum (Fig. 3 Separations 1 and 2) and between the cerebellum, cortex and medulla of the guinea-pig (Separations 7, 8 and 9). Thus human cerebral cortex yielded some ten components, while the cerebellum yielded eight. The major differences were seen in proteins migrating towards the anode which in the case of the cortex contained three components not present in the cerebellum. A major feature of the human specimens was the dense band (X) with a mobility similar to serum albumin, which was almost completely absent from the extracts of pigeon and mammal brain. Differences of an equally marked type were found in different regions of guinea-pig brain though the protein fractions involved were distinctly different from those of human cerebral tissues (c.f. Fig. 3 Separations 1, 2 and 3).

It must be noted that only proteins of a size or charge enabling them to enter the gel will be detected. Others of high molecular weight either remain fixed on the

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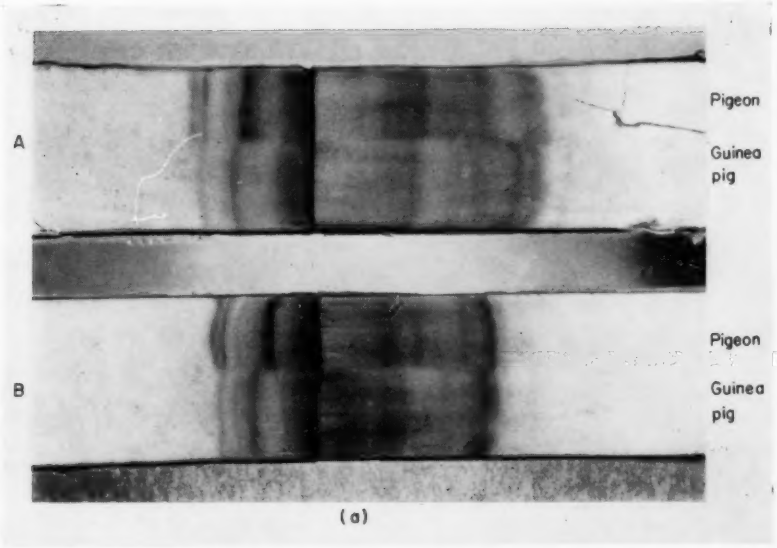

FIG. 1.—Photograph of starch gels after electrophoresis of soluble proteins of guinea pig and pigeon whole brain.

Upper gel (A) made with 0·04 M-borate buffer, pH 8·3. Lower gel (B) made with 0·025 M-borate buffer, pH 8·3.

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paper used to introduce the sample, or enter the gel only slowly and form the densely staining masses near the origin.

It was noted in many experiments with guinea-pig brain that the pattern of the protein bands obtained was not always identical, variations in the number and position of certain bands frequently occurring. Since it is well established that the presence or absence of certain serum proteins, as detected by starch gel electrophoresis is genetically determined (SMITHIES, 1955, 1958; SMITHIES and WALKER, 1955, 1956; ASHTON, 1957, 1958*a*, *b*), it was considered that similar differences may well extend to proteins of the brain. Experiments were carried out with four inbred strains of rats,

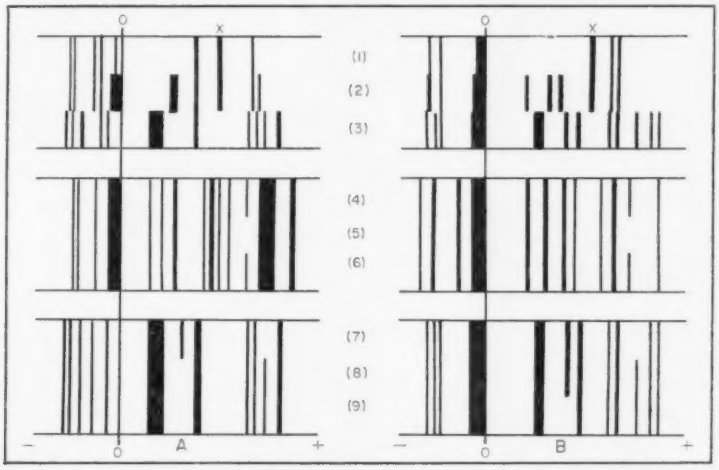

FIG. 3.—Diagrammatic representation of starch gels after electrophoresis of soluble proteins extractable from different parts of the brain of man, ox and guinea pig.
Gels in Column A made with 0·025 M-borate buffer, pH 8·3. Gels in Column B made with 0·04 M-borate buffer, pH 8·3. 'O' indicates the point of insertion of the sample. (1) Human cerebellum, (2) human cortex, (3) guinea-pig cortex, (4) ox cerebellum, (5) ox cortex, (6) ox thalamus, (7) guinea-pig cerebellum, (8) guinea-pig cortex, (9) guinea-pig medulla.

two of which had been selected on the basis of different emotional reactivity to a standard stress situation (BROADHURST, 1958), and three homozygous strains of mice supplied by Professor H. GRUNEBERG. Although no differences were detectable in the cerebral proteins from the rats, those from the mice revealed definite qualitative differences in certain minor components (Fig. 2, Separations 5, 6 and 7). In these experiments, brains from three mice of each strain were pooled to provide sufficient material for electrophoresis. Two such experiments were run, using mice from different litters in each case. The results from both experiments were identical.

DISCUSSION

The results presented above show that upon fractionation of cerebral proteins in a starch gel, the number of components detectable in the proteins forming the cytoplasm are greater than those previously found by other methods. Both electrophoresis on paper and electrophoresis in an agar gel have commonly revealed the presence of some 7–9 components in cerebral extracts (LE BARON, 1959). Thus, the demonstration of up to 15 separable components by the new technique represents a

considerable improvement in the degree of resolution. This greater resolving power of the starch gel has enabled differences to be clearly seen, not only between proteins from different species but also between proteins from different parts of the same brain. Previously, paper electrophoresis has been stated to reveal no differences between cerebral proteins extracted from different species (Tonini and Missere, 1957; Hofmann and Schinko, 1956; Karcher *et al.*, 1959), to show changes in quantity in similar extracts (Polyakova, 1956), or to show differences in mobility according to the region of the brain from which they were extracted (Keup, 1955). Such lack of agreement probably reflects both the difficulties of achieving adequate separations and differences in the methods chosen to extract various types of proteins.

The method of extraction chosen in the present work, similar to that employed by Caravaglios and Chiaverini (1956), Pelligrino and Caravaglios (1957), and Karcher *et al.* (1959), would appear to be particularly suitable for obtaining proteins specific to the cytoplasm. In addition, the technique of concentration by pervaporation is considered to be a milder method than is freeze-drying. Indeed, attempts to freeze-dry extracts made in aqueous sucrose and dialysed against distilled water (Caravaglios and Chiaverini, 1956) yielded a residue part of which was not soluble in the buffer systems employed. The soluble fraction showed a markedly altered behaviour on subsequent electrophoresis.

Owing to the lack of an adequate scheme of nomenclature, it is difficult to attempt a classification of the individual fractions in terms of serum proteins. This is the more so since serum proteins were poorly separated under the conditions optimal for cerebral proteins. Nevertheless at least one fraction (Fraction X in Fig. 2) possessed a mobility similar to that of serum albumin, and three bands possessed mobilities much greater. The presence of a faint band corresponding to albumin in, say, extracts of guinea-pig cortex, must always give rise to suspicion of contamination by serum, particularly since the intensity of such a band could be raised when small quantities of serum were added to the extract. The low intensity of this band in extracts from guinea-pig and rat would thus agree with the statement (Palladin, 1957) that albumin is absent from animal brain. On the other hand, in extracts from human cortex or cerebellum (Fig. 3) electrophoresis revealed the presence of a considerable quantity of a component of a mobility similar to serum albumin. Values of between 15–25 per cent of the total protein have been recorded for a component of similar mobility in human tissues (Kiyota, 1959; Lowenthal *et al.*, 1959).

A major finding of the present work is the demonstration of the differences existing between the proteins of different species and from different parts of brain and comment has been made above. Such differences may indicate differences in the cell types from which the proteins are extracted, or may be a reflection of the different functions of the various regions. However, implications of this nature must be decided by more direct means and attempts to obtain such information on these and related topics are being actively pursued.

SUMMARY

(1) The technique of electrophoresis in a starch gel medium has been modified for the separation and examination of proteins from cerebral tissues.

(2) Cytoplasm proteins have been extracted in aqueous isotonic sucrose and concentrated by pervaporation before electrophoresis. After separation the proteins

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were stained by Naphthalene Black 10B. By this means some 15 components were distinctly recognisable.

(3) It has been shown that distinct qualitative differences exist in the protein patterns obtained from the whole brain of different species, and from different parts of the brain of a single species. Human brain contains an appreciable quantity of a component with the same mobility as serum albumin.

(4) Examination of the proteins from the whole brains of three genetically pure strains of mice revealed qualitative differences in the proteins of the whole brain. No differences were detected in proteins from the whole brain of inbred strains of rat.

(5) The results are discussed in relation to the greater resolving power of the starch gel medium.

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NEUROCHEMICAL CORRELATES OF BEHAVIOR*—II

CORRELATION OF BRAIN MONOAMINE OXIDASE ACTIVITY WITH BEHAVIOURAL CHANGES AFTER IPRONIAZID AND 5-HYDROXYTRYPTOPHAN ADMINISTRATION†

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WHEN brain levels of 5-hydroxytryptamine (5-HT)‡ are experimentally elevated in animals by injecting its precursor, 5-hydroxytryptophan (5-HTP), the animals exhibit behavioural disturbances (UDENFRIEND *et al.*, 1957; BOGDANSKI *et al.*, 1958). The resulting atypical behaviour has been described in subjective terms. In other experiments of this type, numerical ratings of the visual observations have been made in dogs (COSTA *et al.*, 1959; HIMWICH, 1960). Recent studies from our laboratories have established the quantitative relationship between the dose of intramuscularly administered 5-HTP and its behavioural effects by employing operant (learned behaviour) techniques (APRISON and FERSTER, 1960,1961). The same authors have also quantitatively measured the enhanced behavioural effects of 5-HTP following iproniazid administration (FERSTER and APRISON, 1959). In the present study, we correlated the behavioural effects of 5-HTP with brain monoamine oxidase (MAO) activity. The correlation was made over a wide range of brain MAO (9–100 per cent normal) by pretreating the birds with iproniazid (*iso*propyl-*iso*nicotinyl hydrazide).

EXPERIMENTAL

Monoamine oxidase activity. The COTZIAS and DOLE (1951) method for measurement of liver MAO activity was adapted to brain tissue. This method involves the measurement of NH_3 with the micro-diffusion technique. The enzyme, prepared from whole brain as an homogenate (30 mg/ml), was assayed in porcelain Conway vessels at 29·8 ± 0·2°, pH 7·2 (0·2 M-phosphate buffer), and final substrate (tyramine) concentration of 10^{-2} M. Incubation time was 2 hr. Appropriate homogenate and substrate blanks were also carried out.

Behavioural techniques. Pigeons (White Carneaux Cocks) approximately 6 months old were used in this study. The hungry birds were trained to perform in a Skinner box. The behaviour was sustained and recorded by rewarding the hungry bird with food when it pecked at an illuminated disk (key). An electrical contact on the key recorded each instance of the behaviour and made it possible to record and programme this portion of the entire experiment remotely and automatically. The pigeon's response was rewarded intermittently to provide a reproducible pattern of behaviour which the bird repeated continuously over the 6-hour experimental sessions (FERSTER and SKINNER, 1957). When injected compounds disrupted the birds' performances severely, the session was prolonged until the birds completed their normal number (55) of reinforcements. The base line used to determine the

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† Paper I of this series is Reference 2 of this paper.

‡ The abbreviations used are: 5-HT, 5-hydroxytryptamine (serotonin); 5-HTP, DL-5-hydroxytryptophan hydrate; MAO, monoamine oxidase.

behavioural effects of the injected 5-HTP was a multiple fixed-interval, fixed-ratio schedule (mult FI 10 FR 50) which produced a predictable performance pattern related to the bird's disposition to perform. The multiple schedule base line has been used to assess the behavioural effects of a variety of CNS drugs (DEWS, 1956, 1958; VERHAVE, 1959; BOREN and NAVARRO, 1959) and CNS metabolites (FERSTER and APRISON, 1959; APRISON and FERSTER, 1960, 1961).

Injection procedure. Injections were made after the reinforcement at the end of the third fixed interval when the colour behind the key was red and the schedule of reinforcement was FR 50. At this point, the injection *per se* has the least effect on the bird's performance, since the fixed-ratio is more resistant to disruption than the fixed-interval schedule. The injections occurred at approximately 31·5 min after the start of the experiment (30 min plus the time for the three fixed ratios).

5-HTP was dissolved in a minimal amount of dilute HCl. This solution was adjusted to pH 7·3 ± 0·1 with a few drops of dilute NaOH and diluted with water so that the final injection volumes were kept to approximately 0·5 ml. Comparable volumes of saline had no effect on the behaviour. Intramuscular injections were made in the same region of the bird's breast muscle. Iproniazid injections were made in the same way.

The data were recorded on graphic cumulative recorders, digital display counters, and digital printing counters.

RESULTS

The behavioural effect of an intramuscular injection of 50 mg/kg of 5-HTP in normal pigeons was similar to the results already reported (APRISON and FERSTER, 1960, 1961). When the same birds were also given 50 mg/kg doses of iproniazid several days after recovery from 5-HTP administration they were not affected, i.e. their behaviour was identical to that noted on control days when either saline was injected or no injection was made.

Data from a typical experiment (Fig. 1) show the enhanced behavioural effect of 5-HTP after iproniazid pretreatment. Curve 1 describes the effect of the first 50 mg/kg 5-HTP injection given 1 hour after the iproniazid treatment. The bird stopped responding immediately after the injection and did not peck for the remainder of the experimental session, approximately 10 hours long. On the day following this dose, the bird's behaviour returned to normal and is included within the stippled band. Each subsequent day, the pigeon's performance was normal until the next time 5-HTP was injected. The performance after the second 50 mg/kg 5-HTP injection (5 days after the iproniazid pretreatment) was again atypical for the entire experimental session (Curve 2). The period during which the bird did not respond at all was somewhat less than that occurring after the first 5-HTP injection. The bird began responding before the end of the session but at a reduced rate. Further injections of 50 mg/kg 5-HTP were given on 12, 15, 20, 26, 28, 31, 35, 39, and 47 days following iproniazid administration. Representative performances from these periods are shown by Curves 3, 4, 5, 6, 7 and 8. These data show the gradual return of the daily performances to the pre-iproniazid level. Within each experimental session, the recovery of responding was gradual. The actual terminal rate of responding after the birds had recovered from the 5-HTP increased over the terminal rate of responding in the 5-HTP and saline control sessions.

Visual observation during the period of atypical or depressed responding showed that the pigeon was either immobile or shifting his weight rapidly from one leg to another. In addition, there were irregular brief periods of rapid body and head tremors.

The data from Fig. 1 are replotted in a simpler form in Fig. 2 to show the potentiation of behavioural effects of 5-HTP by iproniazid. This form is normally used for

enzyme recovery curves (Fig. 3). The behavioural effect was expressed as the reciprocal of the atypical or depressed responding time ('T') during which the bird's performance was disrupted by the injection of 5-HTP. After the first 5-HTP injection following the iproniazid treatment, the behavioural effects fell to about one-fourth of the control value. Thereafter, the bird's performance gradually recovered, reaching maximal values of 1/'T' at about 35 days. The 1/'T' values larger than the control

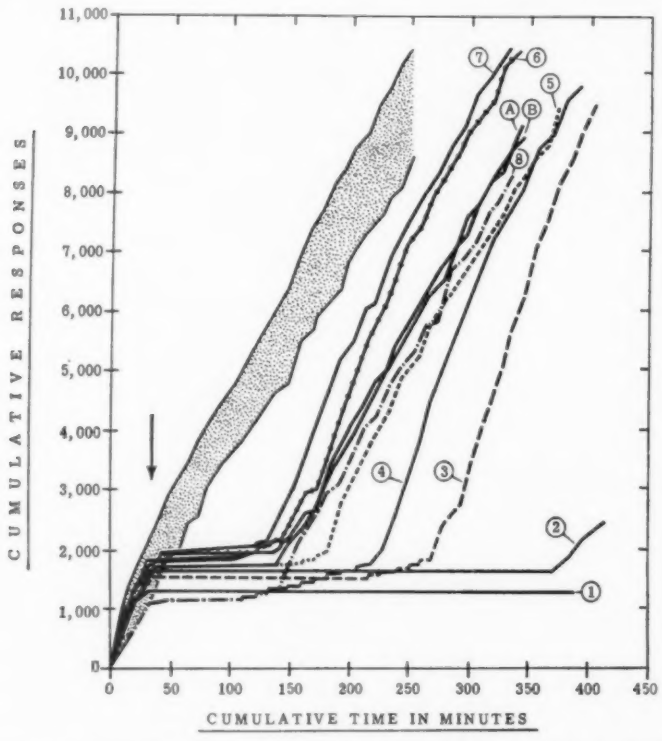

FIG. 1.—Performance curves of the pigeon showing the effect of a 5-HTP injection (50 mg/kg) at various times following iproniazid pretreatment. Pigeon was injected with iproniazid in three consecutive 50 mg/kg doses equally spaced over a period of approximately 40 hours. One hour after the last iproniazid injection, a 50 mg/kg dose of 5-HTP was administered intramuscularly; then at different times over a 47 day period, the same dose of 5-HTP was given without any further iproniazid administration. The performance is recorded cumulatively as the number of pecks the bird makes plotted against time. The stippled band at the left represents the range of all the control data, including saline injections. The three iproniazid injections had no behavioural effects and are also included in the stippled band. The arrow indicates the point in the time sequence at which injections were made. Curves A and B describe the typical behavioural effect of 5-HTP when administered prior to any iproniazid treatment. The remaining curves were recorded at subsequent times during the 47-day recovery from the iproniazid injection (see text).

occurring between the 35th and 47th day are probably due to the bird's accommodation to repeated doses of 5-HTP rather than an above-normal increase of MAO activity during this period (see Fig. 3).

The brain MAO activity, after iproniazid pretreatment (Fig. 3), fell to about 9 per cent of normal 1 hour after the last injection. It then increased until normal values were reached at approximately 42 days. Liver MAO activity in the same birds

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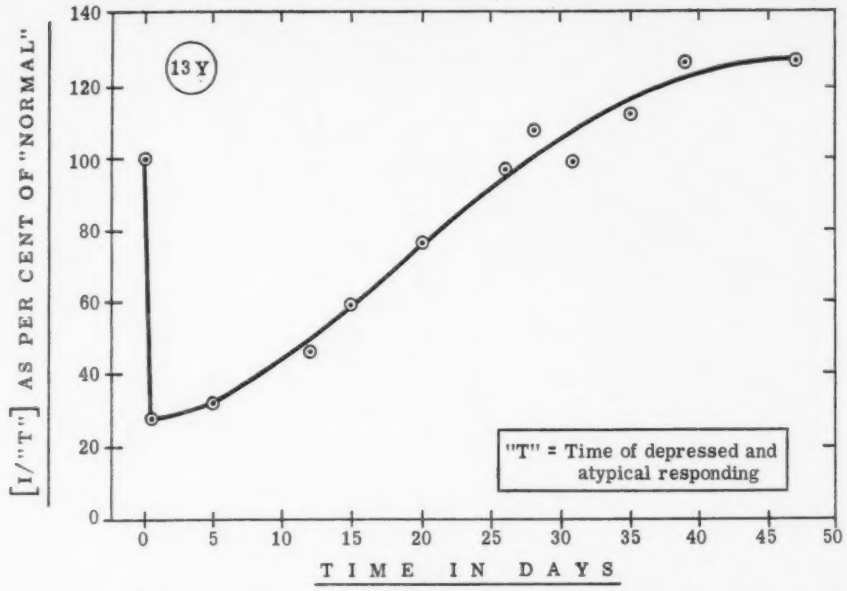

FIG. 2.—Potentiation of the effect of 5-HTP by iproniazid pretreatment. The curve expresses the behavioural effect, 1/'T', of a constant 5-HTP injection periodically given during 47 days following iproniazid treatment.

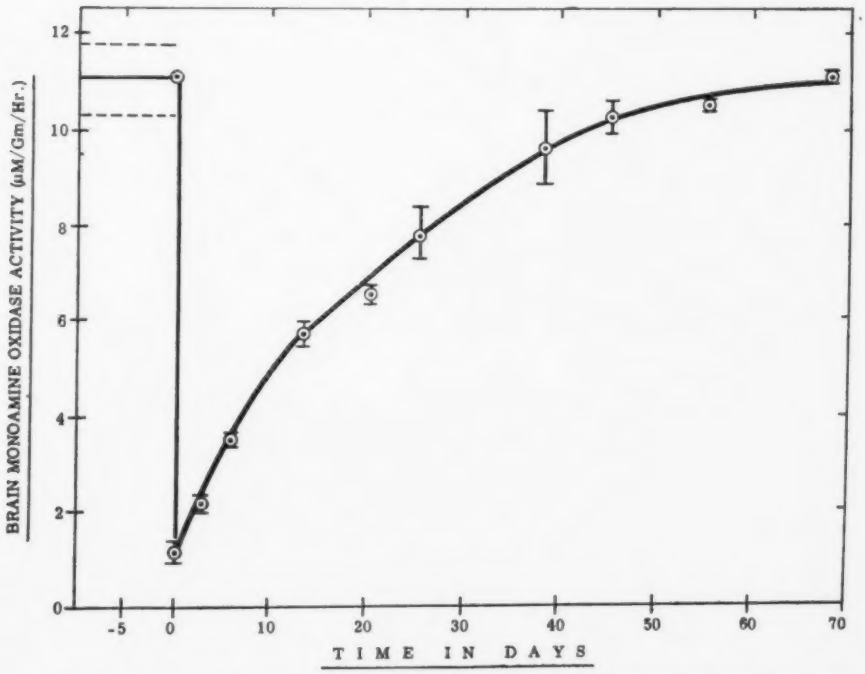

FIG. 3.—Recovery of brain monoamine oxidase activity after iproniazid. Each of 32 pigeons was given the same iproniazid treatment as in the 5-HTP experiments and sacrificed at the indicated time.

(the 45 and 68-day pigeons were not determined) returned to normal in less than half this time (Table 1).

The data presented in Fig. 4 for Pigeon 13Y correlate the behavioural effects of

TABLE 1.—RECOVERY OF LIVER MAO ACTIVITY IN PIGEONS PRETREATED WITH IPRONIAZID (THREE 50 mg/kg DOSES)*

Time (days)	Liver MAO activity (μmoles NH_3/g per hr)	
	Individual values	Mean
0	36·4, 33·7, 16·2, 15·0, 21·8, 23·2	24·4
0·06	0·30, 0·30, 0·30	0·3
3	5·7, 6·9, 15·3	9·3
6	13·8, 15·7	14·8
13	28·8, 29·4, 24·3	27·5
20	25·7, 19·7, 20·2	21·7
25	19·7, 22·6	21·1
40	25·0, 24·0, 24·0	24·3
55	21·8, 19·2	20·5

* Recovery time started after the third injection of iproniazid.

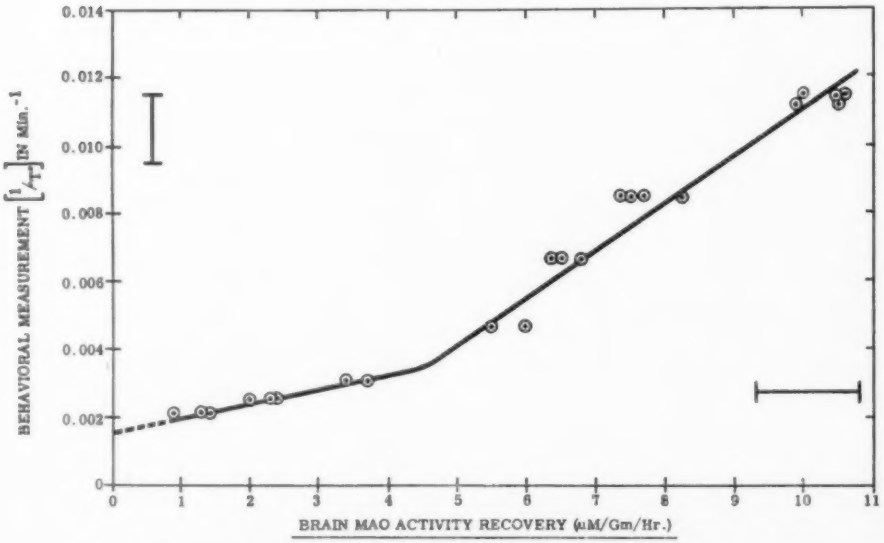

FIG. 4.—Correlation of the behavioural effect of 50 mg/kg of 5-HTP with the brain MAO activity current at the time of the injection. The range of the behavioural measurement and brain MAO activity are given in the upper left and lower right-hand part of the figure respectively. The behavioural measurement was expressed as 1/'T', the reciprocal of the time during which the bird's performance was depressed or atypical. Brain MAO activity was expressed as μmoles NH_3/g per hr.

repeated 5-HTP doses (50 mg/kg) with brain MAO level present at that time. A similar plot has been obtained for another bird. The ordinate values of each point are derived from behavioural measurements of the enhanced effect of injected 5-HTP after iproniazid pretreatment similar to those shown in Fig. 2. The abscissa values

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were derived from the independent measurements of brain MAO activities described in Fig. 3. Even though the MAO data are from birds other than the one whose performance is being measured, the interbird variability is small enough for the MAO values to be reliably extrapolated. The behavioural effect of the injected 5-HTP at normal brain MAO activity is given by the range indicated in the upper left-hand

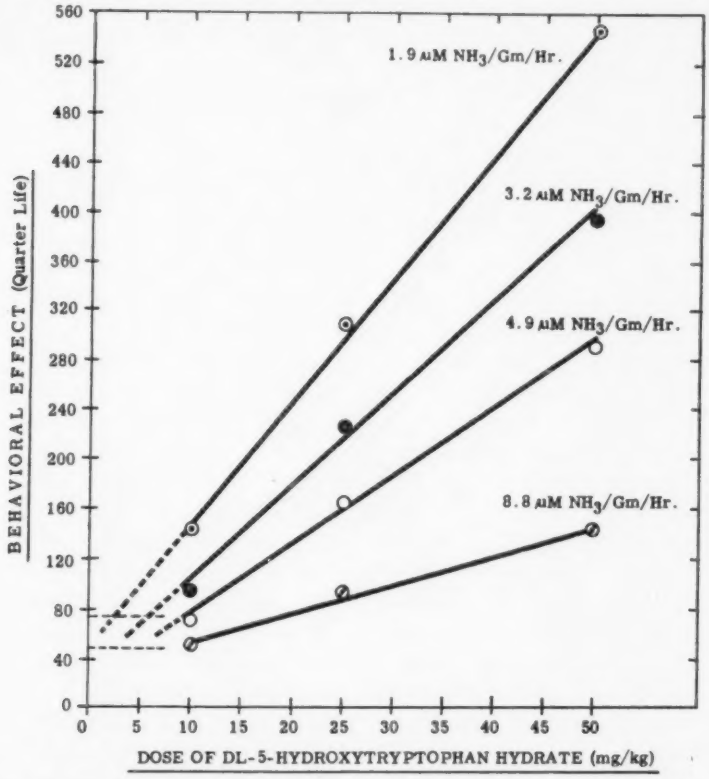

FIG. 5.—The behavioural effect of varying doses of 5-HTP at constant levels of MAO activity. Curves are given at four values of MAO activity ranging from abnormally low levels to near normal levels. Each point on the curve represents the mean value for two pigeons. The birds were pretreated with iproniazid.

corner of the Figure. The normal brain MAO levels are indicated by the range given in the lower right-hand corner of the Figure. As the brain MAO activity increased, so did the behavioural measure, the slope of the curve being greater at the higher enzyme levels.

In similar experiments in which pigeons were pretreated with three 50 mg/kg doses of iproniazid, the effect of injecting 10, 25, or 50 mg/kg doses of 5-HTP was also studied. It was thus possible to plot the behavioural effect against the dose of 5-HTP, with brain MAO activity as a parameter (Fig. 5). In these experiments the behavioural effect of injected 5-HTP was expressed as the quarter-life of the cumulative-response curve: the time necessary for the bird to emit one-fourth the total number of responses made in the session. This type of behavioural measurement was developed to overcome a difficulty of the ('T') measurement when the pigeon's rate

of pecking slowly returns to normal. With this type of performance, the point in time at which the depressed or atypical responding returns to normal can be ambiguous. However, the quarter-life measurement is not influenced by any such subjective factor.

At each dose of 5-HTP, the greatest behavioural effect (quarter-life) was obtained at the lowest brain MAO activity (Fig. 5). The slopes of the curves decrease with a rise in brain MAO activity. These data indicate a lesser behavioural effect of 5-HTP as the brain MAO levels recover.

DISCUSSION

These experiments show a quantitative relationship between the change in the behaviour of a pigeon injected with iproniazid and 5-HTP, and the change in its brain MAO activity. In addition, the data suggest that the behavioural changes seen in the bird are due to changes in brain serotonin, the latter's concentration being controlled by the level of MAO. The correlation was possible because techniques have been developed to measure quantitatively the performances of the pigeons under controlled conditions. The reproducibility of the behavioural base line was comparable with that obtained in the usual biochemical and pharmacological bioassay techniques. As a result of such advances, it is now possible to measure quantitatively the performance or behaviour of an animal objectively, and in turn correlate these changes with biochemical and physiological parameters.

Our explanation of the behavioural effect has been in terms of the serotonin level in brain. This hypothesis was suggested by the work of many investigators (GADDUM, 1954; WOOLLEY and SHAW, 1954, 1957; MARRAZZI and HART, 1955; BRODIE and SHORE 1957; UDENFRIEND *et al.*, 1957; COSTA and APRISON, 1958). Since a constant amount of 5-HTP (50 mg/kg) is injected at various times during the 47-day period following iproniazid pretreatment, the observed change in behaviour should be due to the action of cerebral serotonin formed from it. During this period, brain MAO activity is slowly returning to normal (Fig. 3). Therefore, as the activity of this enzyme rises, more of the free neurohumor is destroyed, and hence less is available to produce its behavioural and physiological effects. There is an implicit assumption in this discussion that 5-HTP and iproniazid have their behavioural effect by permitting the production of varying amounts of free brain serotonin; therefore, the MAO levels are directly, though inversely, correlated with the neurohumor levels.

This hypothesis is further strengthened by the data shown in Fig. 5. At lower brain monoamine oxidase levels, the same dose of 5-HTP produces its greatest behavioural effect. This, of course, is understandable from the explanation of the behavioural effect in terms of the free serotonin levels present in brain. The non-linearity of the form of the correlation between the behavioural effect and MAO activity is probably due to the presence of sufficient enzyme to destroy the free serotonin at a more efficient rate when some critical level of brain MAO activity is reached. This, of course, could result in a different rate of depletion of brain serotonin at higher MAO levels and produce the curvature in the form of the correlation curve (Fig. 4). In addition, it should be remembered that the free serotonin levels and MAO activity vary inversely. Therefore, the form of the correlation curve between behaviour and brain MAO activity would suggest that a similar type of relationship should exist between free serotonin and brain MAO activity, i.e. it might not be a straight-line

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function but a curvilinear one. Such an analysis predicts that at lower doses of 5-HTP following the same iproniazid pretreatment the relation between the behavioural change and the MAO change would be more linear in form (less curvature). This is indeed the case when the data in Fig. 5 are replotted with the behavioural effect as ordinate and brain MAO activity as abscissa.

SUMMARY

The behavioural effect of 50 mg/kg DL-5-hydroxytryptophan hydrate injected intramuscularly was quantitatively measured in pigeons with operant conditioning techniques. The behavioural effect of 5-HTP was markedly enhanced when the birds were pretreated with three 50 mg/kg doses of iproniazid equally spaced over a period of 40 hours. Similar doses of 50 mg/kg of 5-HTP were given at various times during a period of 47 days and the enhancement of the behavioural effect decreased toward pre-iproniazid values. The behavioural change was correlated with the recovery of brain monoamine oxidase activity during this period and was explained in terms of the free serotonin present in the brain after 5-HTP injections.

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UPTAKE OF AMINO ACIDS BY MOUSE BRAIN SLICES

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AMINO acids injected into the bloodstream enter the brain only slowly (GREENBERG and WINNICK, 1948; SCHWERIN, BESSMAN and WAELSCH, 1950; KAMIN and HANDLER, 1951; DINGMAN and SPORN, 1959; RICHTER, 1959) although taken up readily by many other tissues. Investigations using labelled methionine (RICHTER, 1959) or glutamic acid (LAJTHA, BERL and WAELSCH, 1959) confirm that these amino acids pass from the blood into the brain slowly, but show that on entering the brain they are rapidly involved in metabolic activity. A blood–brain barrier might account for the observed facts.

In metabolic experiments interference by a blood–brain barrier may be avoided by the use of brain slices. By this means the uptake of glutamic acid has been investigated *in vitro* by STERN, EGGLESTON, HEMS and KREBS (1949), who showed that the glutamic acid concentration in brain slices was raised above that of the medium in which the slices were suspended. More recently TSUKADA, NAGATA and HIRAMO (1960) have shown that this can also occur with γ-aminobutyric acid.

A number of free amino acids (MCILWAIN, 1959) including glutamic acid and γ-aminobutyric acid (CRAVIOTO, MASSIEU and IZQUIERDO, 1951) are found in brain at concentrations greater than 1 m-mole/kg tissue weight, whereas in blood their concentrations are in most cases much less than that figure. However, many amino acids are present in brain at concentrations of 0·2 m-mole/kg or less (SCHURR, THOMPSON, HENDERSON, WILLIAMS and ELVEHJEM, 1950), a figure not very different from their concentrations in the blood. It would be of interest to know whether the ability of brain to take up amino acids against a concentration gradient is confined only to those free amino acids present in brain at concentrations greater than 1 m-mole/kg, or whether it also includes those amino acids which are present in brain at very low concentrations.

Experiments have been carreid out *in vitro* on six of the amino acids which are present in brain in the free state at concentrations less than 0·2 m-mole/kg (including proline, lysine and methionine, whose concentrations in the brain appear to be slightly less than in the blood). They show that brain tissue is not unlike other tissues (WISEMAN, 1955; WISEMAN and GHADIALLY, 1955) in that it can take up against a concentration gradient *in vitro* all six amino acids investigated.

The effect of different experimental conditions on the uptake of histidine by brain *in vitro* has also been demonstrated.

Although experiments *in vivo* suggest that brain is different from other tissues as regards the uptake of amino acids, experiments *in vitro* indicate that it is basically similar and that the blood–brain barrier probably prevents the accumulation of a number of amino acids by brain *in vivo*.

METHODS

Experimental procedure. Mice weighing between 25 and 35 g were killed by dislocation of the neck. After decapitation the cranium was removed and the brain divided transversely, level with the posterior borders of the cerebral hemispheres. The portions of the brain anterior to the line of division were transferred to a glass platform and sliced as thinly as possible by hand, using a small quantity of bicarbonate saline as lubricant. The brain slices so produced from one brain were placed in a 150 ml Erlenmeyer flask containing 20 ml of bicarbonate saline medium (KREBS and HENSELEIT, 1932), saturated with a gas mixture of 5% CO_2 in 95% O_2, containing 0·3% glucose and the amino acid under investigation. The air in the flask was replaced with a gas mixture of 5% CO_2 in 95% O_2 and the flask tightly stoppered. The flask and its contents were then shaken at constant temperature by the use of a Warburg bath (rate of shaking 50 oscillations/min, amplitude 8 cm). (Never more than 2 min elapsed between the death of the mouse and the start of the shaking.) After a measured time in the bath (referred to as the incubation period) the tissue and about 5 ml of the suspending medium were transferred to a round-bottomed siliconed centrifuge tube (9·5 cm × 1·7 cm) and centrifuged for 10 min at 3000 r.p.m. in a centrifuge with built-in windshield and swing-out heads (distance from rotor to tip of glass tube 14·1 cm). After centrifugation the supernatant fluid was decanted, the centrifuge tube dried and surplus fluid removed from the surface of the tissue by means of filter paper. The tube and contents were then weighed, thereby obtaining the wet weight of the tissue together with adherent medium. The tissue was resuspended in a measured volume of water and homogenized in an all-glass homogenizer. The protein in the homogenate was precipitated in 5% trichloroacetic acid and the filtrate collected. The protein in a measured sample of the supernatant fluid collected after centrifugation was also precipitated in 5% trichloroacetic acid and the filtrate collected. A specific colorimetric method was then used to determine the amount of amino acid in the filtrate.

Amino acid solutions. The amino acids (all of the L-form) were of A or CfP grade obtained from the California Corporation for Biochemical Research, Los Angeles. Histidine, lysine and arginine were used as the monohydrochlorides.

Chemical estimations. Amino acids were estimated by colorimetric methods using a Unicam (Cambridge) S.P.500 spectrophotometer. Proline, lysine and ornithine were determined by the method of CHINARD (1952) using a wavelength of 515 mμ. Methionine was determined by the method of MCCARTHY and SULLIVAN (1941) modified by using 0·4 ml of 14·3 N-NaOH and 1·0 ml of the HCl–H_3PO_4 mixture instead of the recommended volumes, making up to 10 ml with distilled water and reading at a wavelength of 510 mμ. Histidine and arginine were determined by the methods described by MACPHERSON (1946), using a wavelength of 500 mμ in both cases. Recovery of added arginine from brain tissue was found to be 112 per cent and results have been corrected accordingly.

Control experiments. Experiments were carried out with no amino acid in the suspending medium in order to determine in tissue and suspending medium the apparent amino acid concentration produced by material reacting with the colorimetric reagents. The apparent amino acid concentration of each of the amino acids in the suspending medium at the end of the experimental period was found to be less than 0·01 mM.

Experiments using cyanide as inhibitor and experiments under anaerobic conditions were also carried out. In both cases an incubation period of 30 min and a temperature of 37° were used, with 5 mM histidine in the suspending medium. Cyanide was used as 5 mM neutralized KCN. Anaerobic conditions were obtained by using 5% CO_2 in 95% N_2 in place of the 5% CO_2 in 95% O_2 gas mixture.

pH determinations. pH was determined using the Radiometer (Copenhagen) pH meter model PHM 23.

Presentation of results. The accumulation of amino acid in the tissue is presented in the form of concentration ratio developed, that is, the ratio of the concentration of amino acid in the tissue (as weighed) to the concentration in the suspending medium at the end of the incubation period. The figures given are the mean and standard deviation; the latter was calculated using the formula for small samples. No correction has been made for adherent medium, and the specific gravity of the tissue has been taken as 1·00.

RESULTS

The results shown in Table 1 indicate that, when suspended for 30 min in a medium which contains an amino acid at a concentration of 5 mM, brain slices can take up that

TABLE 1.—AMINO ACID CONCENTRATION RATIOS DEVELOPED BETWEEN BRAIN SLICES AND SUSPENDING MEDIUM

Amino acid	Number of brain samples	Concentration ratio developed
Histidine	8	5·09 ± 0·33
Proline	12	2·76 ± 0·15
Ornithine	16	1·87 ± 0·13
Methionine	16	1·70 ± 0·30
Lysine	12	1·66 ± 0·10
Arginine	11	1·34 ± 0·12

Initial amino acid concentration in suspending medium 5 mM. Incubation 30 min at 37°.

TABLE 2.—APPARENT AMINO ACID CONCENTRATIONS IN BRAIN TISSUE SLICES AFTER INCUBATION IN SUSPENDING MEDIUM CONTAINING NO ADDED AMINO ACID

Amino acid	Number of brain samples	Apparent tissue concentration (m-moles/kg tissue weight)
Methionine	15	1·23 ± 0·68
Lysine	6	0·77 ± 0·08
Proline	6	0·30 ± 0·03
Histidine	6	0·28 ± 0·07
Ornithine	6	0·25 ± 0·03
Arginine	6	0·16 ± 0·09

Incubation 30 min at 37°.

amino acid and produce a tissue concentration of the amino acid higher than its concentration in the suspending medium. The concentration ratio developed was greatest for histidine and least for arginine. The small amount of amino acid already present in the tissue may be ignored, as the apparent tissue concentrations of amino acids using no amino acid in the suspending medium (Table 2) were considerably lower than the 5 mM concentration in the medium.

Different experimental conditions using histidine altered the concentration ratio developed (Figs. 1, 2 and 3; Table 4).

The concentration ratio found after a longer incubation period was higher than that after a shorter one (Fig. 1), the highest mean value using 2 mM histidine in the suspending medium being 12·4 for the maximum time of 90 min. The lowest value found was 0·53 for zero time, which represents an experimental procedure omitting the placing of the flask in the Warburg bath. The incubation period was not extended beyond 90 min in view of the possibility that the volume of suspending medium might then be insufficient to maintain optimum metabolism (MCILWAIN, 1959).

The concentration ratios developed after 30 min using different concentrations of histidine in the suspending medium are shown in Fig. 2. The maximum mean concentration ratio attained was 8·47, when 0·5 mM histidine was used, although this concentration ratio is not significantly different from that developed when 0·25 or 1·0 mM

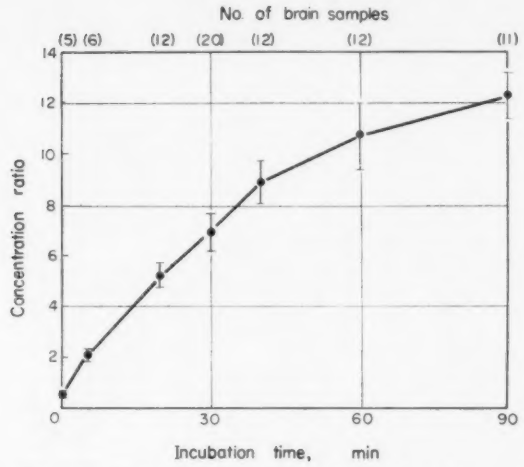

FIG. 1.—Effect of incubation time on uptake of histidine by brain slices. Concentration ratio shown as mean with standard deviation. Initial concentration in medium 2 mM. 37°.

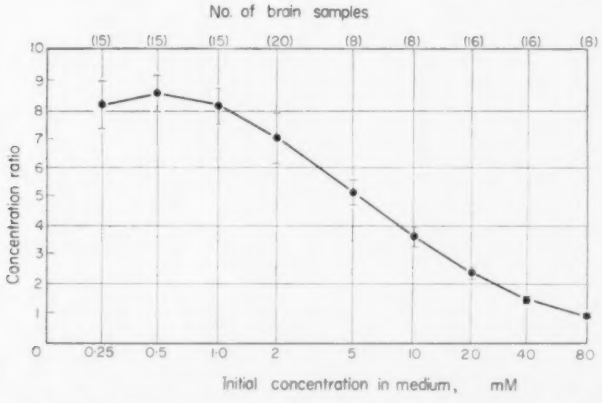

FIG. 2.—Uptake of histidine by brain slices using different concentrations of histidine in suspending medium. Concentration ratio shown as mean with standard deviation. Incubation 30 min at 37°.

histidine was used. As the concentration of histidine in the suspending medium was made greater, so the concentration ratio produced fell. With the highest concentration of histidine used, 80 mM, the concentration ratio had fallen to 0·91, in other words the concentration of histidine in the tissue had not reached that in the suspending medium by the end of the incubation period.

It was found that the pH of the suspending medium was lowered when the concentration of histidine (hydrochloride) in it was 10 mM or greater (Table 3). It was therefore considered that the low concentration ratios produced when concentrations greater than 5 mM were used might be due partly to the acidity of the medium, and so experiments were carried out using 5 mM histidine to determine the effect of pH on the concentration ratio developed. The results are set out in Table 4 and it is clear that when the pH is altered from normal the concentration ratio is lowered.

TABLE 3.—pH OF SUSPENDING MEDIUM CONTAINING DIFFERENT CONCENTRATIONS OF HISTIDINE (HYDROCHLORIDE)

Initial concentration of histidine (mM)	pH	
	before incubation	after incubation
5	7·2	7·5
10	6·9	7·3
20	6·5	6·9
40	6·0	6·2
80	5·7	5·7

Incubation 30 min at 37°.

TABLE 4.—EFFECT OF pH ON HISTIDINE CONCENTRATION RATIO DEVELOPED BETWEEN BRAIN SLICES AND SUSPENDING MEDIUM

pH of suspending medium		Number of brain samples	Concentration ratio developed
Initial	Final		
7·8	8·0	7	4·34 ± 0·23
7·2	7·5	8	5·09 ± 0·33
6·6	7·0	6	3·95 ± 0·26
6·1	6·3	7	3·27 ± 0·25
4·4	5·2	8	1·56 ± 0·12

Initial concentration of histidine in suspending medium 5 mM.
Incubation 30 min at 37°.

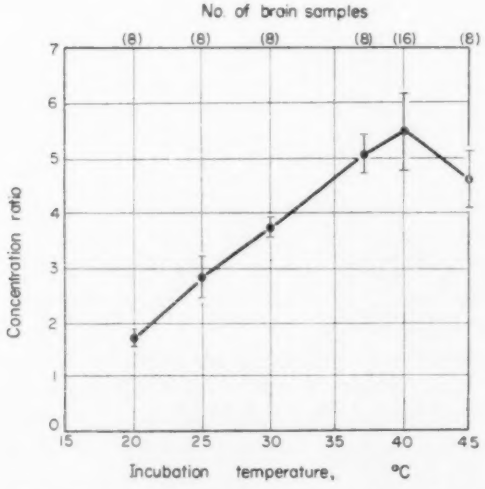

FIG. 3.—Effect of temperature on uptake of histidine by brain slices. Concentration ratio shown as mean with standard deviation. Initial concentration of histidine in medium 5 mM. Incubation period 30 min.

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However, if the suspending medium containing 40 mM or 80 mM histidine was neutralized with added sodium bicarbonate, the uptake of histidine by the tissue was not significantly altered when compared with results obtained using similar concentrations with the medium not neutralized.

The effect of temperature on the uptake of histidine by brain tissue was also investigated (Fig. 3). The concentraion ratio developed was a maximum when the temperature was 40°.

The ability of brain tissue to take up histidine (using a 5 mM concentration in the medium) was greatly reduced in the presence of cyanide (concentration ratio 1·05 ± 0·09, N = 6) and under anaerobic conditions (concentration ratio 1·24 ± 0·06, N = 6).

DISCUSSION

It is clear that brain is not unlike other tissues (CHRISTENSEN and RIGGS, 1952; CHRISTENSEN, RIGGS and RAY, 1952; CHRISTENSEN and STREICHER, 1949; RIGGS, CHRISTENSEN and PALATINE, 1952; WISEMAN, 1955; WISEMAN and GHADIALLY, 1955) in that it has considerable capacity for taking up amino acids against a concentration gradient.

TABLE 5.—COMPARISON BETWEEN CONCENTRATION RATIO DEVELOPED BY BRAIN TISSUE AND SARCOMA TISSUE *in vitro*

Tissue	Number of tissue samples	Histidine concentration ratio*
Mouse brain	7	3·42 ± 0·29
Sarcoma RD3†	15	3·94 ± 0·48

16 mM-L-histidine in suspending medium.
Incubation 1·5 hr. at 37°.

* Tissue concentrations calculated in terms of tissue water.
† From WISEMAN and GHADIALLY (1955).

The only other tissues in which there have been investigations into the transport *in vitro* of most of the amino acids used in the experiments described here are sarcoma (WISEMAN and GHADIALLY, 1955) and intestinal tissue (WISEMAN, 1955). An attempt has been made to compare the uptake of histidine by brain with its uptake by sarcoma tissue (Table 5), using 16 mM-L-histidine in the suspending medium, although a direct quantitative comparison must be interpreted with caution owing to differences in technique, etc. The main differences between the experiments seem to be (i) differences in centrifugation (results should not be affected by more than 2 per cent), (ii) differences in amount of adherent medium, (iii) the presence of extracellular fluid in tissue from brain (DAVSON and SPAZIANI, 1959) whereas the sarcoma tissue was in the form of a cell suspension, and (iv) damage to the cellular elements of brain tissue. Using the technique described above, a mean concentration ratio of 3·42 was developed by brain tissue for histidine, whereas WISEMAN and GHADIALLY, using a somewhat similar technique, found that sarcoma tissue produced a mean concentration ratio of 3·94. These results indicate that brain and sarcoma tissue take up histidine *in vitro* to a similar degree.

Brain tissue also appears to be not unlike sarcoma tissue in respect to its degree of preference for taking up amino acids. Of the amino acids investigated, both tissues

accumulate histidine to the greatest degree, with proline next. Ornithine, lysine and methionine are also taken up by both tissues against a concentration gradient, but to a lesser degree.

The other tissue on which a study of these particular amino acids has been made is intestinal tissue (WISEMAN, 1955), which differs from both brain and sarcoma tissue in that it transports proline the most actively, but transports neither lysine nor ornithine against a concentration gradient. However, the investigations on intestinal tissue were concerned with movement of amino acids through the wall of the intestine, rather than uptake by cells.

It is interesting to compare the concentration ratio developed using histidine with that which might be developed using glutamic acid under somewhat similar conditions. By extrapolating from the results of STERN *et al.* (1949) it appears that, using 5 mM-glutamic acid for an incubation period of 30 min at 40°, brain tissue would develop a glutamic acid concentration ratio of about 5·4. The mean concentration ratio using 5 mM-histidine under similar time and temperature conditions (Fig. 3) was found to be 5·51. The comparison, again, is not as direct as might at first appear, as the experimental techniques were not identical. The energy requirements would also be different as the initial high concentration of glutamic acid in brain tissue gave a concentration ratio of 3·85 at zero time whereas the tissue concentration of histidine at zero time was such as to give a mean concentration ratio of 0·53 (Fig. 1).

The effect on the uptake of histidine of alterations in time of incubation, temperature and pH is generally that which might be expected. That the concentration ratio developed is affected by differences in the concentration of amino acid in the suspending medium (Fig. 2) has also been shown in the case of proline for intestine (WISEMAN, 1955) and of glutamic acid for brain (STERN *et al.*, 1949). 40 mM and 80 mM-histidine (hydrochloride) in the suspending medium lowered the pH of the medium considerably, but as neutralization with bicarbonate in these cases did not significantly affect the concentration ratio produced, a low pH probably plays only a negligible part in the production of the low ratios. It is more likely that the low values are due either to a direct inhibition of the concentrating mechanism by high histidine concentrations, or else to limitation of the rate of diffusion, or to both.

Inhibition by cyanide and by anaerobic conditions of the uptake of histidine by brain suggests that the concentrating mechanism in brain slices is dependent upon energy production, as appears to be the case with uptake of amino acids by diaphragm (CHRISTENSEN and STREICHER, 1949), intestine (AGAR, HIRD and SIDHU, 1954), reticulocytes (RIGGS *et al.*, 1952) and carcinoma tissue (CHRISTENSEN and RIGGS, 1952), but not with erythrocytes (CHRISTENSEN *et al.*, 1952).

The finding that brain has the capacity to accumulate a number of amino acids in addition to glutamic acid and γ-aminobutyric acid suggests that this capacity might cover all or most of the other amino acids not investigated. The fact that quantitatively brain appears to be not unlike sarcoma tissue in respect to histidine uptake, also suggests the possibility of quantitative similarities with other tissues.

Brain is unlike many other tissues in that resting levels of many free amino acids in the fresh brain are lower than the corresponding blood levels (SCHURR *et al.*, 1950). Other tissues maintain levels of free amino acids higher than the corresponding blood levels, presumably by means of the concentrating mechanism for amino acids of the cell. In the case of brain, the blood–brain barrier appears effectively to prevent similar

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uptake of amino acids from the blood into brain cells. It is not unlikely that a mechanism in the brain for the uptake of amino acids is essential for normal brain function (although the possibility that it may be to a great extent redundant must not be ignored). It is suggested that it is necessary for clearing from the extracellular space, shown to be present by DAVSON and SPAZIANI (1959), amino acids some of which, namely 5-hydroxytryptophan (PAGE, 1958), 3:4-dihydroxyphenylalanine (PELLERIN, LEDUC and D'IORIO, 1958) and glutamic acid (AWAPARA, LANDUA, FUERST and SEALE, 1950) appear to be precursors of probable transmitter substances in brain. 5-Hydroxytryptophan (BOGDANSKI, WEISSBACH and UDENFRIEND, 1959) and 3 : 4-dihydroxyphenylalanine (CARLSSON, LINDQVIST and MAGNUSSON, 1957) have been shown to produce central stimulation when injected into the peripheral bloodstream. It is also possible that other amino acids may be related to as yet unidentified transmitter substances. It is suggested that any deficiency in a concentrating mechanism for amino acids in brain might, by allowing precursors to leak into the extracellular space, affect the neurotransmitter mechanism of the brain, and hence affect normal brain function.

SUMMARY

(1) The uptake of the L-forms of histidine, proline, lysine, ornithine, methionine and arginine by mouse brain tissue *in vitro* has been studied.

(2) Brain slices were able to take up against a concentration gradient all six amino acids.

(3) The accumulation of histidine by brain tissue was affected by time of incubation, temperature, pH and concentration of histidine in suspending medium, and inhibited by anaerobic conditions and by the presence of cyanide.

(4) The results indicate that brain is not unlike other tissues in possessing a mechanism for accumulating amino acids.

(5) The possible significance of the concentrating mechanism as applied to brain tissue is discussed.

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ANNOUNCEMENT

Seventh International Congress of Neurology
(Rome, 10–15 September 1961)

THE Seventh International Congress of Neurology will be held in Rome, September 10–15, 1961, under the auspices of the World Federation of Neurology and of the National Institute for Nervous Diseases and Blindness, of Bethesda.

Topics for the scientific sessions include "Neurological disorders in porphyria, phenylketonuria and galactosuria", "Neurological disorders related to liver diseases", "Brain disturbances associated with cardiopulmonary disorders" and "Aphasia".

A few Symposia will be held as well, among which a particularly important one is that of Neurochemistry, which will deal with "Lipids, Lipoproteins and their Metabolism" and with "Changes in Myelin". A Symposium on Neurological Genetics will be held jointly with the Third International Conference of Genetics (Rome, 7–12 September 1961). The other Symposia will deal with Neurological Geography, History of Neurology and Multiple Sclerosis.

The fourth day of the Congress will be dedicated to communications on major topics and free communications, which will be selected by the Congress Secretariat. Communications, whether related to a major topic or not, will be published or summarized in precirculated booklets provided they reach the Office of the Secretary General of the Congress on or before the 15 March, 1961, in triplicate text with an English summary of 500 words.

The Congress will have three categories of membership: *Active member* open to members of the constituent national neurological societies; *Associate member*, open to qualified physicians and scientists having an interest in the Congress; *Adjunct member*, open to interested non-professionals and to families of members of the Congress. Registration fees are $15.00 (or equivalent in Italian lire) for Active Members; $10.00 for Associate members, $5.00 for Adjunct members.

Information is available from the President of the Congress, Prof. MARIO GOZZANO or from the Secretary General, Dr. GIOVANNI ALEMÀ, Viale Università 30, Rome. For the Symposium of Neurochemistry, information should be asked from Dr. ARMAND LOWENTHAL, Secretary for Neurochemistry, World Federation of Neurology, 59, rue Philippe Williot, Berchem-Anvers (Belgium). Dr. JAMES L. O'LEARY, M.D., Washington University School of Medicine, 600 S. Kingshighway, St. Louis, 10, Mo., will issue forms, receive registration applications and fees, and transmit communications to the Secretary General.

The Fifth International Congress of Electroencephalography and Clinical Neurophysiology will be held at Rome from the 7th to the 13th September 1961.

Information is obtainable from the President of the Congress, Prof. MARIO GOZZANO, or from the Secretary General, Dr. RAFFAELO VIZIOLI, Viale Università 30, Rome.

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Papers to be published in future issues

JERZY KRAWCZYNSKI: The influence of serotonin D-lysergic acid diethylamide and 2-brom LSD on the incorporation of [^{35}S] methionine into brain proteins and on the level of ATP in the brain.

SHEILA N. PAYNE and B. S. PLATT: The quantitative separation and estimation of glycosphingosides in nervous tissue, with especial reference in gangliosides.

S. H. CARTER and W. E. STONE: Effect of convulsants on brain glycogen in the mouse.

HUNTINGTON PORTER and SALLY AINSWORTH: The intracellular distribution of copper in brain.

GUY M. MCKHANN and DONALD B. TOWER: The regulation of γ-aminobutyric acid metabolism in cerebral cortex mitochondria.

F. S. BAILEY and P. J. HEALD: Quantitative estimation of proteins in extracts of cerebral tissue after separation by electrophoresis in starch gel.

OLE J. RAFAELSEN: Action of insulin on carbohydrates of isolated rat spinal cord.

OLE J. RAFAELSEN: Action of insulin of glucose uptake of rat brain slices and isolated rat cerebellum.

OLE J. RAFAELSEN and T. CLAUSEN: Fate of glucose in isolated rat spinal cord and diaphragm incubated in the absence and presence of insulin.

JEAN R. HOLBROOK: Effects of alterations in serum copper levels upon the liver, basal ganglia and cerebellar cortex in the guinea pig.

ALLEN C. CROCKER: The cerebral defect in Taysachs disease and Niemannpick disease.

Y. TAKAHASHI and Y. AKABANE: Incorporation of ^{14}C-glycine into glutathione in rat cortex slices.

Y. TAKAHASHI, M. NOMURA and S. FURUSAWA: *In vitro* incorporation of ^{14}C-amino acids into proteins of peripheral nerve during Wallerian degeneration.

RYO TAKAHASHI, TOSHIO NASU, TAKEO TAMURA and TETSUHIKO KARIYA: Relationship of ammonia and acetylcholine levels to brain excitability.

ROBERT KATZMAN and CLARENCE E. WILSON: Extraction of lipid and lipid cation from frozen brain tissues.

R. S. DE ROPP and E. H. SNEDEKER: Effect of drugs on amino acid levels in the rat brain: hypoglycemic agents.

A. LOWENTHAL, M. VAN SANDE and D. KARCHER: Heterogeneity of lactic and malic dehydrogenase in cerebrospinal fluid.

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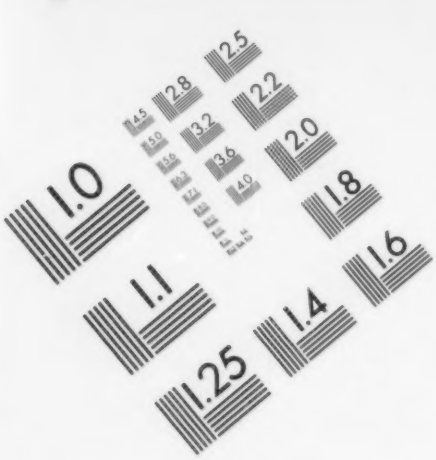

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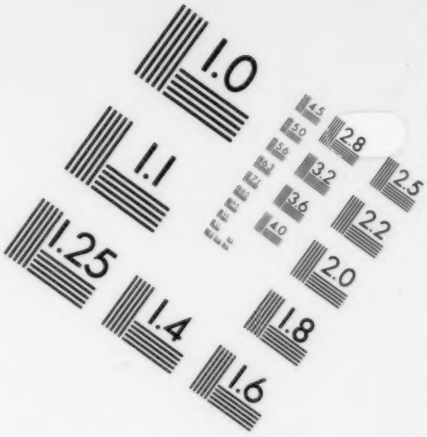

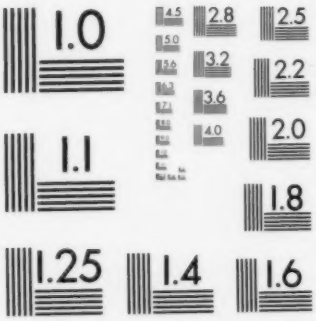

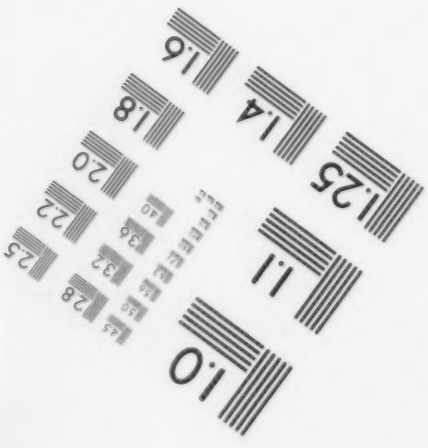

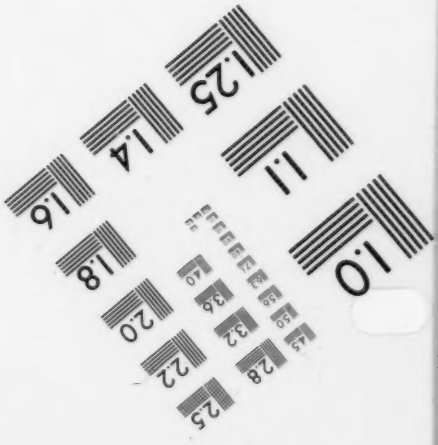

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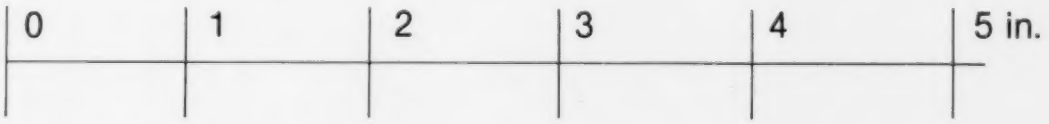

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